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UNRESTRICTED

TOWARDS THE ABSOLUTE QUANTIFICATION OF DNA BY PCR

A thesis submitted in partial fulfilment of the requirements of the Open University
for the degree of Doctor of Philosophy

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August 1999

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ABSTRACT

Amplification techniques such as the Polymerase Chain Reaction (PCR) are held to be largely qualitative procedures and are widely used as such. Since the efficiency of amplification is less than perfect, small changes in efficiency can yield dramatic differences in the final amount of product generated. Despite this unpredictability the exquisite sensitivity of PCR makes the demanding goal of absolute quantification highly desirable. Consequently, the use of this technique for the quantification of nucleic acids has increased at an exponential rate. However, the ability of PCR to accurately quantify absolute levels of DNA is still not universally accepted.

The overall aim of this investigation was to determine the critical factors affecting the quantification of DNA using PCR and to use these findings to develop an assay for the absolute quantification of DNA in a model system. The novel work presented here illustrates the need for careful examination of sequences for GC-rich domains which could give rise to stable secondary structures and reduce the efficiency of amplification by serving as termination sites.

To determine the accuracy of competitive PCR, CE and IP-RP-HPLC were employed to quantify PCR products. These two techniques provided valuable information on the identification and elimination of sources of error which led to improvements in speed, accuracy and precision, as well as ease of quantification by PCR. They also yielded information on the process of heteroduplex formation whilst simultaneously revealing assay limitations.

Consequently, the on-line fluorescence monitoring of PCR was used as an alternative method for the quantification of *Legionella pneumophila*. This technique was highly reproducible however, mispriming and the subsequent amplification of non-specific PCR products limited the level of detection. The 3'-end labelling of degraded DNA with DIG prevented short DNA fragments from mispriming (and consequently extending) allowing the amplification of DNA targets. Therefore, to reduce mispriming and hence improve assay sensitivity, this approach was adapted for the first time to produce 5'-degenerate, 3'-DIG-terminated competitive primer analogues. These analogues, coupled with the use of the Lightcycler™, allowed the detection and absolute quantification of a single cell of *Legionella pneumophila*. This is the first time that this level of sensitivity has been achieved using this type of assay.

This technique should provide a very rapid and sensitive alternative for quantification compared to the other, more expensive technologies available at present.

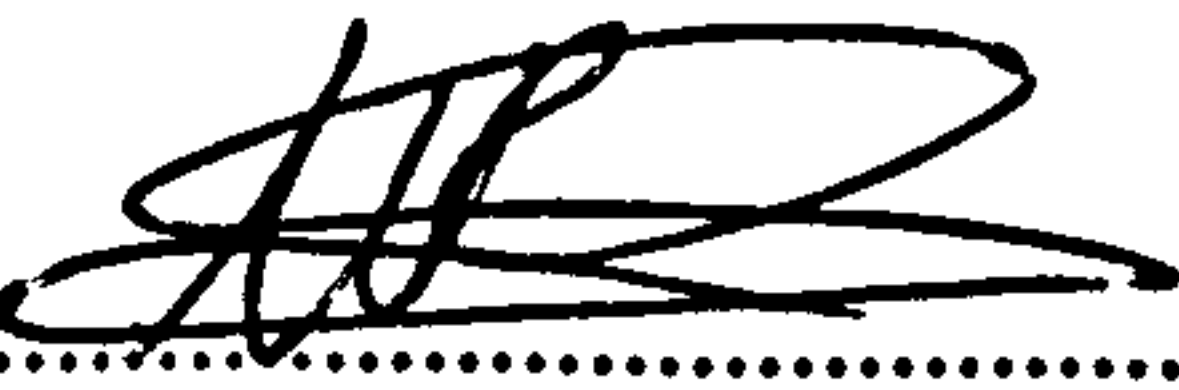
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I declare that the work presented in this thesis is the result of my own investigations, except where otherwise stated. This work has not previously been accepted for any degree and is not being concurrently submitted in candidature for any degree. I hereby give consent for my thesis, if accepted, to be available for photocopying and for interlibrary loan, and for the title and summary to be made available to outside organisations.

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PUBLICATIONS

McDowell, D.G., Burns, N.A. and Parkes, H.C. (1998). Localised sequence regions possessing high melting temperatures prevent the amplification of a DNA mimic in competitive PCR. *Nucleic Acids Research* **26**, 3340-3347.

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ABBREVIATIONS

A	adenine
A ₂₆₀	absorbance at 260 nm
A ₂₈₀	absorbance at 280 nm
ABI	Applied Biosystems International
bp	base pair
BSA	bovine serum albumin
C	cytosine
CE	Capillary electrophoresis
cPCR	competitive PCR
c.p.m	counts per minute
CTAB	cetyltrimethylammonium bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DIG	digoxigenin
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate

EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
G	guanine
HPLC	High performance liquid chromatography
HWM	high molecular weight
IAA	isoamyl alcohol
kb	kilobase
LIF	laser-induced fluorescence
MWM	molecular weight marker
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	Perkin Elmer
QPCR	quantitative PCR
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SSC	salt sodium citrate
ssDNA	single-stranded DNA
T	thymine
TE	tris-EDTA buffer
T _m	melting temperature
TMAC	tetramethylammonium chloride
Tris	tris(hydroxymethyl)aminomethane
U	uracil
UV	ultraviolet

Weights, volumes and concentrations

M	molar
mol	mole
g	gram
L	litre

Prefixes

m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})
f	femto (10^{-15})
a	atto (10^{-18})

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 Introduction

Amplification techniques such as the Polymerase Chain Reaction (PCR) are held to be largely qualitative procedures and are widely used as such, with the level of product generated taken to be independent of initial target concentration. The amplification process is exponential, i.e. the product of one cycle of amplification acting as the substrate for the next. Theoretically the amount of product generated doubles with each round of amplification. However, experimentally, the amplification efficiency, i.e. the fraction of the template replicated during each cycle, is less than perfect. Small changes in efficiency can yield dramatic differences in the final amount of product generated, regardless of the amount of starting material. A multitude of factors can affect the efficiency of the reaction, many in an as yet unpredictable and uncontrollable manner.

Despite this unpredictability, which has led to some doubt regarding the validity of PCR as a quantitative tool, a large body of work published over the last 10 years has documented the development of quantitative PCR (QPCR) protocols designed for use with a range of targets including cellular, viral and bacterial nucleic acids (Ferré, 1992; Ferré, 1998; Kanangat *et al.*, 1992; Kolk *et al.*, 1994; Piatak *et al.*, 1993). The ability of PCR to reproducibly detect tens of molecules within a background of hundreds of millions makes the demanding goal of absolute quantification highly desirable. Existing methods for specific nucleic acid copy number determination, such as Southern blotting and *in situ* hybridisation, are laborious and do not lend themselves to processing large numbers of samples (Ferré *et al.*, 1992). Since the first reports of QPCR (Becker-André and Hahlbrock, 1989; Gilliland *et al.*, 1990), the use of the technique has increased at an

exponential rate. Despite these reports, the ability of PCR to accurately quantify absolute levels of DNA is not universally accepted. In addition, as with other new technologies, the nomenclature and theoretical basis of the technique has become confused. The aim of this review is to attempt to clarify these points and to describe the existing state of the technology currently available for the quantification of DNA. This introduction is therefore intended to provide the background and theoretical basis to the disparate approaches to QPCR so that the rationale for the investigations presented in this thesis can be better understood.

1.2 The Amplification and Quantification of DNA using PCR

Since the advent of PCR in 1986 (Mullis *et al.*, 1986) this technique has been rapidly applied to the quantification of DNA. However, the quantification of DNA by PCR has been hampered by the lack of fast, reliable and accurate methods. Over a decade since its advent however, quantitative PCR technology is still being developed for the absolute quantification of DNA.

1.2.1 The polymerase chain reaction (PCR)

This section is not meant to deal with the specifics of PCR in detail but rather to give a brief overview of the technique (for extensive reviews of the technique see Erlich *et al.*, 1991 and Mullis *et al.*, 1994). PCR is in general a three stage process, with denaturation, annealing and elongation steps. The temperatures employed during these steps vary and are subject to a number of considerations that need to be determined empirically. The number of cycles used for the PCR varies depending on target concentration and the efficiency of the reaction. As will be discussed in Section 1.4.1, it is best to use a cycle number that generates visible products during the exponential, rather than the plateau phase, of the reaction.

Other factors (as reviewed by Erlich *et al.*, 1991) such as the concentrations of $MgCl_2$, DNA polymerase, primers and deoxynucleotide triphosphates (dNTPS), or denaturation,

annealing and extension times can influence the specificity and sensitivity of the reaction. Since magnesium ion concentration and annealing temperature can greatly influence the specificity of the reaction, care must therefore be taken to optimise these parameters. The use of a “hot-start” at the beginning of the reaction may improve performance by eliminating the formation of non-specific products (Mullis, 1991).

1.2.2 The detection of amplification products

The final stage in PCR involves the detection and quantification of amplification products. The reaction products need to be separated so the target and standard can be discriminated and quantified. A large variety of procedures are available for the detection and quantification of amplification products (Jenkins, 1994). The two broad classes of techniques that are currently used for the detection of amplification products are the traditional “end-point” measurements of product and the “real-time” monitoring of product formation. End-point techniques analyse the amount of product generated after the reaction is complete whilst real-time techniques monitor the reaction as it progresses in a thermal cycler. End-point product determination can be accomplished through the use of fluorescent intercalating dyes or through measurement of incorporated radioactivity. Using these methods, PCR products of different sizes are usually separated by conventional gel (agarose or polyacrylamide) electrophoresis (Menzo *et al.*, 1992; Piatak *et al.*, 1993; Zimmermann *et al.*, 1994), capillary electrophoresis (CE) (Fasco *et al.*, 1995; Stålbom *et al.*, 1994) or high performance liquid chromatography (HPLC) (de Kant *et al.*, 1994). The simplest and most commonly used detection method is visualising the DNA bands by ethidium bromide or an equivalent fluorescent dye (Apostolakis *et al.*, 1993; Benavides *et al.*, 1995; Grassi *et al.*, 1994). It was recently reported that the sensitivity of gel electrophoresis detection can be greatly enhanced (10-1000 fold) using the fluorescent dye SYBR Green ITM instead of ethidium bromide (Schneeberger *et al.*, 1995).

For objective quantification of the respective DNA bands, a video analysis system can be used. The gels can be scanned either directly while on the transilluminator (Menzo *et al.*, 1992; Piatak *et al.*, 1993) or after photography (Apostolakos *et al.*, 1993). It must be noted that the amount of incorporated dye and resulting fluorescent emission depends on molar amount of nucleotide present i.e. the amount of dye incorporated is proportional to the length of the product and the mole amount of double-stranded DNA (dsDNA).

Although quantification of very low copy numbers has not been achieved with dsDNA dyes, the simplicity of using these dyes is attractive (Higuchi *et al.*, 1993; Wittwer *et al.*, 1997a). The dyes can be used for any amplification and therefore custom fluorescently-labelled oligonucleotides are not necessary. Quantification of very low copy numbers with dsDNA dyes will require either improved amplification specificity or a means to differentiate the desired product from non-specific amplification.

A procedure more sensitive than ethidium bromide staining, which is also used for the quantification of PCR products, is based on the use of radioactively or fluorescently-labelled dNTPs (Dostal *et al.*, 1994; Furtado *et al.*, 1993) or oligonucleotides (Arnold *et al.*, 1992; Wang *et al.*, 1989). After electrophoretic separation, the radioactively labelled bands can be excised from gels and the c.p.m. determined (Furtado *et al.*, 1993; Scadden *et al.*, 1992). However, drying the gels followed by direct autoradiography and scanning (Dostal *et al.*, 1994) or radioimaging of dried gels (Arnold *et al.*, 1992; Zenilman *et al.*, 1995) is easier and involves less manipulation and is also more precise.

PCR products produced using fluorescently labelled primers can be separated on polyacrylamide gels and the signal quantified using an automated laser fluorescent sequencer. This laser-induced fluorescence (LIF) is a highly sensitive methodology and, in combination with computer software, offers a means of automating post-PCR analysis. LIF can also be applied to the quantification of PCR products following capillary electrophoresis (Fasco *et al.*, 1995; Stålbom *et al.*, 1994). A disadvantage of dNTP direct

labelling is that it also visualises non-specific PCR products whereas the use of labelled primers offers a degree of selectivity and therefore only the required target molecule is detected. However, primers specific for a target can also be incorporated into, and as a consequence direct the synthesis of, non-specific PCR products (Zimmermann and Mannhalter, 1996).

A third type of end-point product measurement uses solid state approaches in which a bound enzyme produces fluorescence or luminescence. One of the mostly widely used formats is a solid-phase capture approach in which the PCR product is sandwiched between capture and reporter systems. After denaturation of the PCR product, the signal is quantified by using either a colorimetric or chemiluminescent detection system. Numerous versions of this sandwich approach have been developed. For instance one group used a DNA probe-coated microwell plate as the capture system and biotinylated primers, combined with colorimetric detection, using avidin-horseradish peroxidase conjugate, as the detection system (Mulder *et al.*, 1994). Although colorimetric systems generally have a poor dynamic range, they expanded the dynamic range of the signal to 4 logs by diluting the amplified products. Chemiluminescent detection is more sensitive than colorimetry and provides a greater dynamic range, eliminating the need to dilute the PCR products prior to quantification. One group reported a dynamic range encompassing 4 logs using such a detection system (Martin *et al.*, 1995).

To achieve more automated signal quantification, methods have been developed utilising this technology (e.g. microplates or magnetic beads) for the quantification of the captured target. The most frequently used procedure involves the amplification of the DNA target in the presence of a biotinylated primer followed by capturing of the PCR products on microplates coated with streptavidin (Jalava *et al.*, 1993; Lehtovaara *et al.*, 1993; Taniguchi *et al.*, 1994). Alternatively, 5'-aminated PCR products can be captured onto carboxylated wells of microplates (Kohaska *et al.*, 1993), or PCR products are captured by probes covalently attached to organosilane plates (Berndt *et al.*, 1995). PCR products

captured in one of the ways described above are hybridised with probes specific for the competitor or target sequences. These probes contain either radioactive labels or have the capacity to interact with enzymes. Quantification is performed by measuring incorporated radioactivity (Jalava *et al.*, 1993), digoxigenin content (Besnard and Andre, 1994; Kohaska *et al.*, 1993) or by immunoenzymatic detection (Lehtovaara *et al.*, 1993; Taniguchi *et al.*, 1994).

Quantitative PCR assays have also been developed for an automated system that makes use of electrochemiluminescence (QPCR System 5000™, Perkin-Elmer, Warrington, UK.) for quantification of PCR products (Heroux and Szczepanik, 1995). One of the primers used for amplification is biotinylated to allow capture of the PCR products by magnetic beads coated with streptavidin. The product signal can be quantified separately by hybridisation with TBR [tris (2, 2'-bipyridine) ruthenium (II) chelate]-labelled probes, specific for the competitor or target sequences, or alternatively TBR-labelled primers are used for direct quantification of the amplified products.

An alternative, sensitive, solid-phase capture system integrated with automated analysis instrumentation has been developed using electrochemiluminescence (ECL) for the quantification of product signal (Katz, 1995). In this assay format, the PCR product is captured onto streptavidin coated magnetic beads and the luminescence results from a chemical reaction that takes place in the electrochemical well. One advantage of this system is that there is no need to manually wash the beads to remove excess probe. Furthermore, this system is extremely sensitive with an estimated detection limit of 10 attomoles. Finally, the system precision claims to be within 5% standard deviation over a linear dynamic range of more than three logs.

Real-time or continuous fluorescence monitoring of the amplification reaction offers the potential for improved quantification because manipulation of the sample for end-point quantification is reduced. Furthermore, a greater amount of information is obtained about

the data points for each cycle. Of the methods currently used for real-time product detection, one takes advantage of the 5' exonuclease activity of *Taq* DNA polymerase through the use of sequence-specific fluorogenic or hybridisation probes (Gibson *et al.*, 1996; Holland *et al.*, 1991; Williams *et al.*, 1998; Wittwer *et al.*, 1998). The second method involves the use of DNA-specific dyes to detect and differentiate between amplification products with differing melting curve profiles (Ririe *et al.*, 1997). This latter approach obviates the need for designing and constructing fluorescent amplification product-specific probes.

1.2.3 Mathematical description of PCR

It is essential to understand the mathematical description of PCR if the full potential of this technique is to be realised. PCR is inherently an indirect method of measurement therefore to derive quantitative data from such a variable system it is necessary to consider mathematical models (Nedelman *et al.*, 1992; Peccoud and Jacob, 1996; Peccoud and Jacob, 1998; Raeymakers, 1993; Wang and Spadaro, 1998). By definition, the PCR is a chain reaction, with the products of one cycle serving as the substrates for the next cycle. Therefore, the amount of product increases exponentially not linearly. Under ideal or theoretical conditions the amount of product doubles during each cycle of the PCR. This can be expressed as in Equation 1:

$$P = T(1+E)^n$$

Equation 1: where P = amount of product detected after n cycles; T = amount of target to be determined; E = amplification efficiency; n = number of cycles through which the PCR proceeds.

As can be seen from Equation 1, small differences in amplification efficiency are compounded exponentially which can lead to dramatic differences in the final product yield of say two initially equal targets. Although it appears at first that the inconsistencies

inherent to PCR preclude its use for accurate quantification, the use of co-amplified standards can control for this potential variability.

1.3 Technical Aspects of Quantitative PCR

1.3.1 Use of standard DNA molecules

Initial reports using quantitative PCR suggested that standards should be used in reactions due to the inherent variability of the technique (Gilliland *et al.*, 1990; Wang *et al.*, 1989). The most widely adopted approach was to co-amplify a standard either in the same or a separate tube. Over the years a wide range of DNA standards have been reported in the literature (reviewed in Ferré, 1992; McCulloch *et al.*, 1995; Zimmermann and Mannhalter, 1996) especially those employed in competitive PCR.

Standards may be endogenous to the sample such as a housekeeping gene or an unrelated exogenous fragment added to the reaction prior to amplification. Endogenous standards are useful for the relative quantification of copy number. These standards have problems of widely different abundances and the use of a different primer pair is required for amplification. Heterologous exogenous standards are an improvement over endogenous standards because the amount of standard added to the reaction can be precisely monitored making it possible to calculate the absolute level of target present in the original sample. The standard controls for variables such as amount and tube to tube variations in amplification efficiency. Quantification of the target is made by comparison of the intensity of the two products. This method only works well if the target and exogenous standard are amplified with similar efficiency, which has to be determined empirically using different combinations of primers. The advantage of using an endogenous standard as an internal standard is that the reference sequence and the target nucleic acid are processed together for the duration of the experiment. This would tend to reduce any differences in the yields of DNA between samples. However, one problem associated with this approach lies in obtaining data before the amplification reactions reach the plateau phase. For example, the internal standard may be present at a much

higher level than the target and amplification of the control may approach plateau phase well before that of the target sequence.

An important requirement of this type of experiment is that the amplification efficiencies for both the target and standard are the same. One of the major problems associated with this type of quantitative PCR lies with the assessment of the reaction's overall efficiency. The absolute amount of product generated does not always bear a consistent relationship to the initial amount of target sequence. The kinetics of each amplification reaction depend not only on the initial amount of target DNA, but also on the length of the products, the priming sequences, potential inhibitors in the test samples and variation of the concentrations of reaction components. Since differences in amplification efficiency are more pronounced in the plateau phase of the reaction the analysis should be carried out during the exponential phase.

Although heterologous exogenous standards have been used successfully for quantification, they are no easier to construct than homologous exogenous standards, consequently there is little advantage gained by their use.

1.3.1.1 Construction of standards for use in quantitative competitive PCR

Standards used in competitive PCR are similar to those used in the previously described approach in that the reference standard is co-amplified in the same reaction as the target of interest. In this case, however, the standard is usually a synthetic template which shares the same primer binding sites as that of the target DNA rather than an exogenous standard which requires the use of an additional primer pair (Becker-André and Hahlbrock., 1989; Gilliland *et al.*, 1990; Siebert and Larrick., 1992).

The construction and testing of a standard, along with the selection of a suitable primer pair, is one of the most crucial and laborious aspects of setting up a competitive PCR protocol. Dieffenbach *et al.*, (1993) have reviewed the general concepts of primer design.

Since He *et al.*, (1994) showed that different primer pairs can exhibit large differences in sensitivity, it would be prudent to test the sensitivity and efficiency of the primer pair before constructing a standard. As mentioned previously, standards used for competitive PCR are DNA fragments that share the same primer binding sites with that of the target sequence and yield products that are distinguishable from the target template. One of the easiest ways to differentiate between the target and standard is by introducing an insert or deletion into the standard so their amplification products are distinguishable by size on a agarose gel. The most widely used method to generate such a standard is to use a composite primer containing two specific target sequences at a predetermined distance from each other (to create a deletion) and a second primer specific for the opposite strand (Celi *et al.*, 1993; Gilliland *et al.*, 1990; Jin *et al.*, 1994; Menzo *et al.*, 1992; Rüster *et al.*, 1995). Amplification of the target template with these primers results in a product shorter than the original target.

If insertions or deletions are required in the middle of the internal standard sequence, a splice overlap technique can be used (Grassi *et al.*, 1994; Porcher *et al.*, 1992; Schanke *et al.*, 1994; Telenti *et al.*, 1992). Using a similar approach, internal standards can also be constructed by amplification of a mixture of religated PCR products (Ali and Steinkasserer, 1995; Galea and Feinstein, 1992). Alternatively, if the sequence of the target template contains one or more restriction sites these can be used to generate a construct with an insertion or a deletion (Arnold *et al.*, 1992; Bruisten *et al.*, 1993; Piatak *et al.*, 1993; Porcher *et al.*, 1992; Xia *et al.*, 1995). It has been shown that the smaller the alteration, the more similar the amplification efficiencies of the target and standard will be (McCulloch *et al.*, 1995). This can be explained by the fact that certain factors can influence the amplification efficiency of a reaction. Of these, the length and sequence of the template amplified, and the sequence of the primers used to amplify the template are important because they effect secondary structure formation (McCulloch *et al.*, 1995). These factors, along with the G/C content of the template, which affects the melting temperature of the target sequence, can interfere with primer binding and/or extension

thus reducing the processivity of the DNA polymerase (Weissensteiner and Lanchbury, 1996). For these reasons, a compromise must be made between the addition/deletion type of standards, where the products are easily distinguishable from one another but are as similar as possible to ensure equal amplification efficiencies.

Differentiation between the target and standard can also be achieved by the incorporation of a unique restriction site (Apostolakos *et al.*, 1993; Becker-André and Hahlbrock, 1989; Fox *et al.*, 1992; Gilliland *et al.*, 1990; Martino *et al.*, 1993; Perrin and Gilliland, 1990; Stieger *et al.*, 1991). After PCR, the amplification products are endonuclease-digested and since the products are smaller in size they can be distinguished from the target template and analysed separately.

Following electrophoretic separation of the standard construct, the standards are usually excised from the gel and extracted (Celi *et al.*, 1993; Jin *et al.*, 1994; Siebert and Larrick, 1993) but more often, they are cloned directly into plasmids (Besnard and Andre, 1994; El-Osta *et al.*, 1999; Menzo *et al.*, 1992; Ramakrishnan *et al.*, 1994; Xia *et al.*, 1995; Zimmermann *et al.*, 1994). This makes it easier to determine the concentration of the standard accurately. Not only should the internal standard be quantified spectrophotometrically but the quantity and quality of the competitive standard should also be checked electrophoretically.

Following construction of the competitive standard, two approaches are available for its use: competitive or non-competitive PCR.

1.3.2 Competitive PCR

Since this technique was first described by Wang *et al.*, (1989) numerous reports have been published on the use of competitive PCR for the quantification of nucleic acids. In general, this technique allows the most accurate quantification of nucleic acids (reviewed extensively in the following references: Ferré, 1992; Clementi *et al.*, 1993; Clementi *et*

al., 1994; Clementi *et al.*, 1995; Siebert and Larrick, 1992). The method uses an exogenously derived internal standard which has the same primer binding sites as that of the target and a similar amplification efficiency, a prerequisite required for an accurate quantitative competitive PCR. This standard is often referred to as a mimic because it has specifically designed similarities to that of the true target. The reaction is truly competitive as the mimic and target sequences are titrated against one another and are co-amplified, so competing for the same primers and therefore amplification.

Typically, a dilution series of either the target or mimic is prepared and a constant amount of either added to each dilution. Although either the target or mimic may be diluted it has to be noted that dilution of the target results in reduced sensitivity. Furthermore, any unknown inhibitors in the target sample will also be diluted. Quantification is performed after amplification of the entire dilution series. From a graph of the log mimic signal/target signal plotted against the log of the initial amount of mimic added, the initial amount of target DNA can be found at the equivalence point (Figure 1.1). However, to derive accurate and reliable results from competitive PCR a number of conditions have to be met. As with any standard graph, the range of standard concentrations used should span the equivalence point. Furthermore, if the amplification products are accurately measured, the gradient of the line obtained should be 1 (Raeymakers, 1995). This slope mathematically represents the ratio of the target and mimic amplification efficiencies, which should be equal in all reactions. In practice though, many methods do not accurately estimate the amount of PCR product, leading to gradients that deviate from the theoretical value. However, as long as the error in estimation is consistent the determination of the equivalence point may not be affected as long as a series of reactions that span the equivalence point are performed. The plot should still, however, be linear. Most reports seem to have neglected these basic tests for mimic constructs. In the absence of such experiments, it is preferable to restrict competitive PCR analysis to the exponential phase of amplification and to consider results as relative rather than absolute (Raeymakers, 1993; Wiesner *et al.*, 1993).

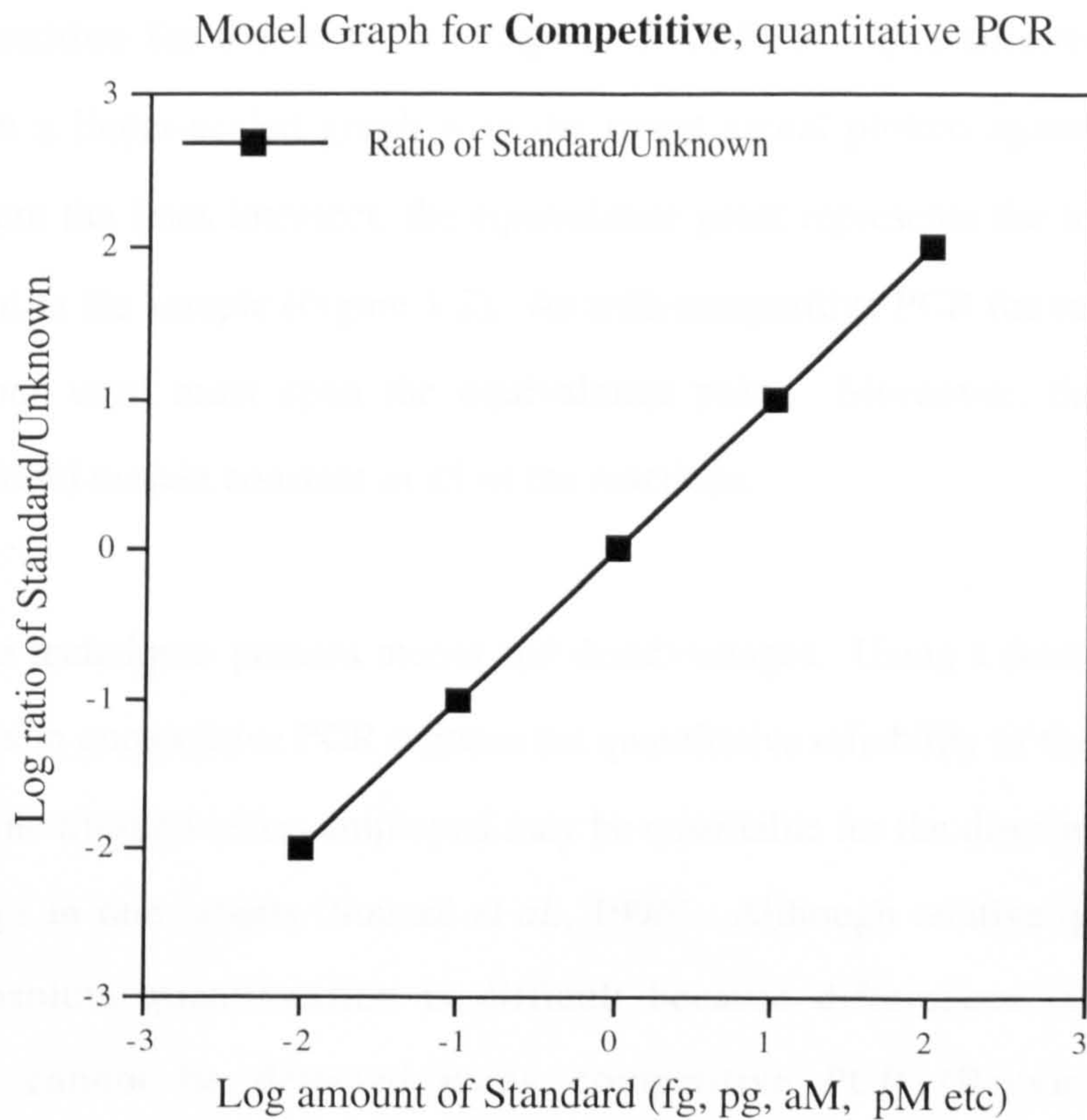


Figure 1.1 Theoretical analysis by competitive, quantitative PCR. A constant amount of target DNA is mixed with increasing concentrations of standard DNA (in this case a range of 5 orders of magnitude). The mixture is subjected to PCR utilising a single set of primers. The most common representation involves plotting log (standard signal/unknown signal) vs. log standard added. When amplification product signals are equal (standard/unknown = 1), the ordinate value will be 0. This should generate a line with a slope of 1.

1.3.3 Non-competitive PCR

Non-competitive PCR differs in the fact that the target signal is unaltered by the presence of a standard. Increasing amounts of the standard are co-amplified in the presence of a constant amount of the target however, this occurs under conditions where there is no actual competition for the reaction components in PCR. Quantification is therefore estimated on a linear-scaled graph with the target signal plotted against the standard signal. Where the lines intersect, the equivalence point represents the initial amount of target present in the sample (Figure 1.2). As with competitive PCR the range of standard concentrations used must span the equivalence point. Moreover, the target signal generated should remain constant in all of the reactions.

Both of these techniques possess merits and disadvantages. Using a double log scale for quantification in competitive PCR reduces the quantitative reliability of the assay because the logarithmic titration series employed may be unsuitable for the discrimination of very small changes in rare targets (Souazé *et al.*, 1996). Although relative quantification is possible, absolute quantification is difficult because differences in amplification efficiencies cannot be detected using competitive PCR (Raeymakers, 1993). Alternatively, since the range of standard employed in non-competitive PCR is small it is necessary to have an approximate idea of the amount of target to be analysed. The greater ability of non-competitive PCR to detect small differences in target product may be better suited to well-characterised systems which detect a narrow range of target. Conversely, competitive PCR is probably better suited to detect large changes in target in less well characterised systems. A combination of these two approaches may be better to use where an initial titration series is first used to determine the approximate concentration of the target and then a smaller range of standards is used for quantification (Nagano and Kelly, 1994; Souazé *et al.*, 1996).

Model Graph for **Non-Competitive** PCR

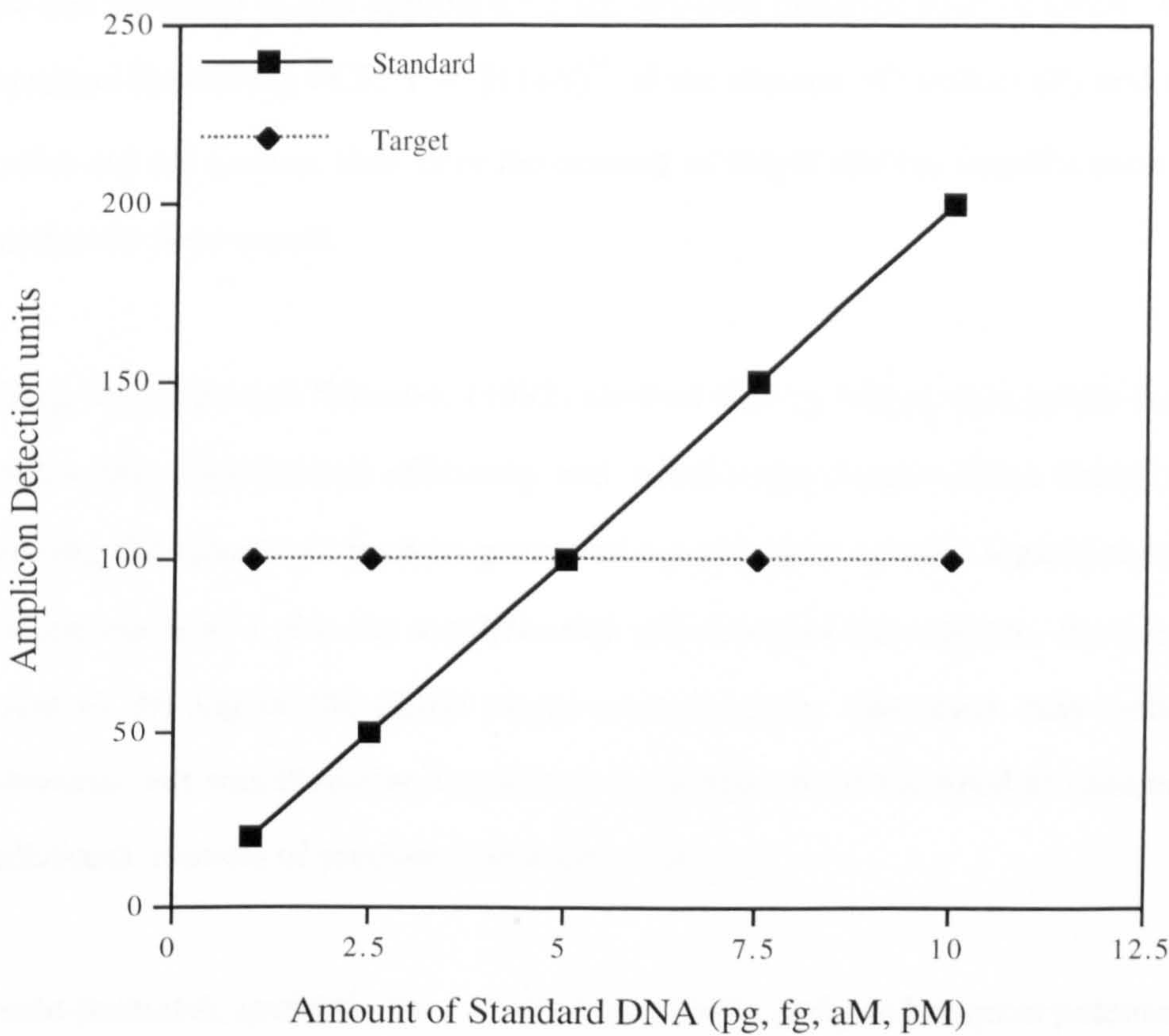


Figure 1.2 Theoretical analysis by non-competitive, quantitative PCR. The DNA target and standard are treated exactly as described in Figure 1.1 with the exception that the standard DNA is added in a linear fashion within one order of magnitude of the target. The point where the standard and target lines cross is the equivalence point and the amount of target present in the sample.

1.3.4 Real-time quantification

The use of amplification kinetics to quantify DNA without the use of mimic was initially employed to avoid the lengthy process of designing, constructing and accurately quantifying the standard itself (Wiesner, 1992; Wiesner *et al.*, 1992). Although this approach was never widely adopted, recent advances in detection methods have revealed the full potential of this approach for the absolute quantification of DNA. From the basic equation describing PCR: $P = T(1+E)^n$, if the amount of product (P) and the number of cycles (n) are known, then only the amount of target and the amplification efficiency (E) need to be determined.

Using this approach Wiesner, (1992) showed that by taking data points from a series of cycles, the amplification efficiency and initial target concentration could be determined. Plotting the amount of product generated against cycle number logarithmically generated a slope equal to 1 plus the amplification efficiency of the reaction, the y-intercept being equal to the log of the initial target concentration. However, this technique proved laborious and was therefore not widely used because of the need to remove and analyse individual aliquots of product after every cycle.

Aside from this, quantification of DNA by kinetic analysis has great potential because the amplification efficiencies of different targets can be determined directly using this technique. Any differences in amplification efficiency are computed and compensated for so no assumptions need to be made concerning variable PCR efficiencies.

Over the last five years, progress in the detection of amplification products has made kinetic quantification possible. Real-time detection of amplification products in the reaction vessel eliminates the need to take aliquots or to use end-point detection techniques. The first developments in this area were made by Higuchi (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993) who monitored the amount of product generated by adding the intercalating dye ethidium bromide to reactions and measuring the fluorescence

emitted. Ethidium bromide fluorescence is enhanced once per cycle during a combined annealing/extension phase when it is intercalated into the dsDNA and is therefore a measure of product concentration. Although this approach had potential, ethidium bromide is a non-specific double-stranded DNA dye thus any fluorescence measurements taken would be from all DNA sources and not just the amplified target. Therefore any system used for the real-time detection of amplification products must be able to differentiate between the target and non-specific product signals.

Of the two systems commercially available which can discriminate amplification product signals, the ABI PRISM[®] 7700 Sequence detection system uses hydrolysis probes (TaqMan[®] probes) to distinguish between specific target and non-specific product signals (Heid *et al.*, 1996; Williams *et al.*, 1998). The probe, added at the beginning of the amplification reaction, allows a direct readout of the amplification since it fluoresces only when it binds to the target sequence. The probe contains two fluorescent dyes, a reporter and a quencher, and is engineered such that the quencher dye inhibits the fluorescence emission from the reporter dye if the probe does not anneal to the target. The reporter and quencher are synthesised on the same oligonucleotide, the quencher dye quenches the fluorescence of the reporter because of the proximity. During temperature cycling, some of the probe hybridises to single-stranded PCR product and may be hydrolysed by the inherent 5' to 3' activity of *Taq* DNA polymerase (Holland *et al.*, 1991; Livak *et al.*, 1995). Because the reporter and quencher molecule are no longer linked, the reporter is released from quenching and the reporter fluorescence increases.

Quantitative data is derived from the determination of cycle number at which the target amplification product first appears (above a predetermined threshold) since the cycle number is proportional to the initial amount of target present in a sample (Gibson *et al.*, 1996; Heid *et al.*, 1996; Williams *et al.*, 1998). More recent developments have made it possible to use intercalating dyes and melting curve analysis.

New, integrated systems such as the LightCycler™ (Idaho Technology Inc., Idaho Falls, USA. and Boehringer Mannheim), which combine an ultra-fast thermal cycler with a micro-volume fluorimeter (Wittwer *et al.*, 1994; Wittwer *et al.*, 1997b), use a double-stranded DNA (dsDNA) dye such as SYBR Green I™ (Higuchi *et al.*, 1993) and fluorogenic hydrolysis or hybridisation probes to monitor the signal in real time. Furthermore, dsDNA dyes allow post amplification melting curve analysis to be performed (Ririe *et al.*, 1997). Continuous monitoring of dye fluorescence throughout the thermocycling profile allows the melting temperature of PCR products to be observed as a rapid decrease in fluorescence at the point of denaturation (Ririe *et al.*, 1997). Since a product's melting curve is reliant on the length, sequence, and GC content of the target, PCR fragments can be differentiated by their melting curves.

Monitoring the amount of product once per cycle with dsDNA dyes is an important advance that allows a wide dynamic range of initial template concentrations to be analysed. The sensitivity is limited at low initial template concentrations because amplification specificity is not absolute. When no template is present, undesirable products such as primer dimers are eventually amplified. This system uses very fast cycle times that increase through put dramatically (Tan *et al.*, 1992; Wittwer *et al.*, 1997b).

1.4 Experimental Considerations for Optimal Performance of QPCR

In addition to the technical considerations discussed previously for the set up of a quantitative PCR system, other concerns that apply to all quantitative methods should be addressed. The reaction phase and heteroduplex formation are all important factors which need to be considered when designing and developing a quantitative PCR protocol.

1.4.1 The reaction phase of PCR

As a PCR progresses, the reaction is seen to go through two distinct phases, the exponential phase and plateau phase (Figure 1.3). Initially the amount of product

PCR Amplification plot

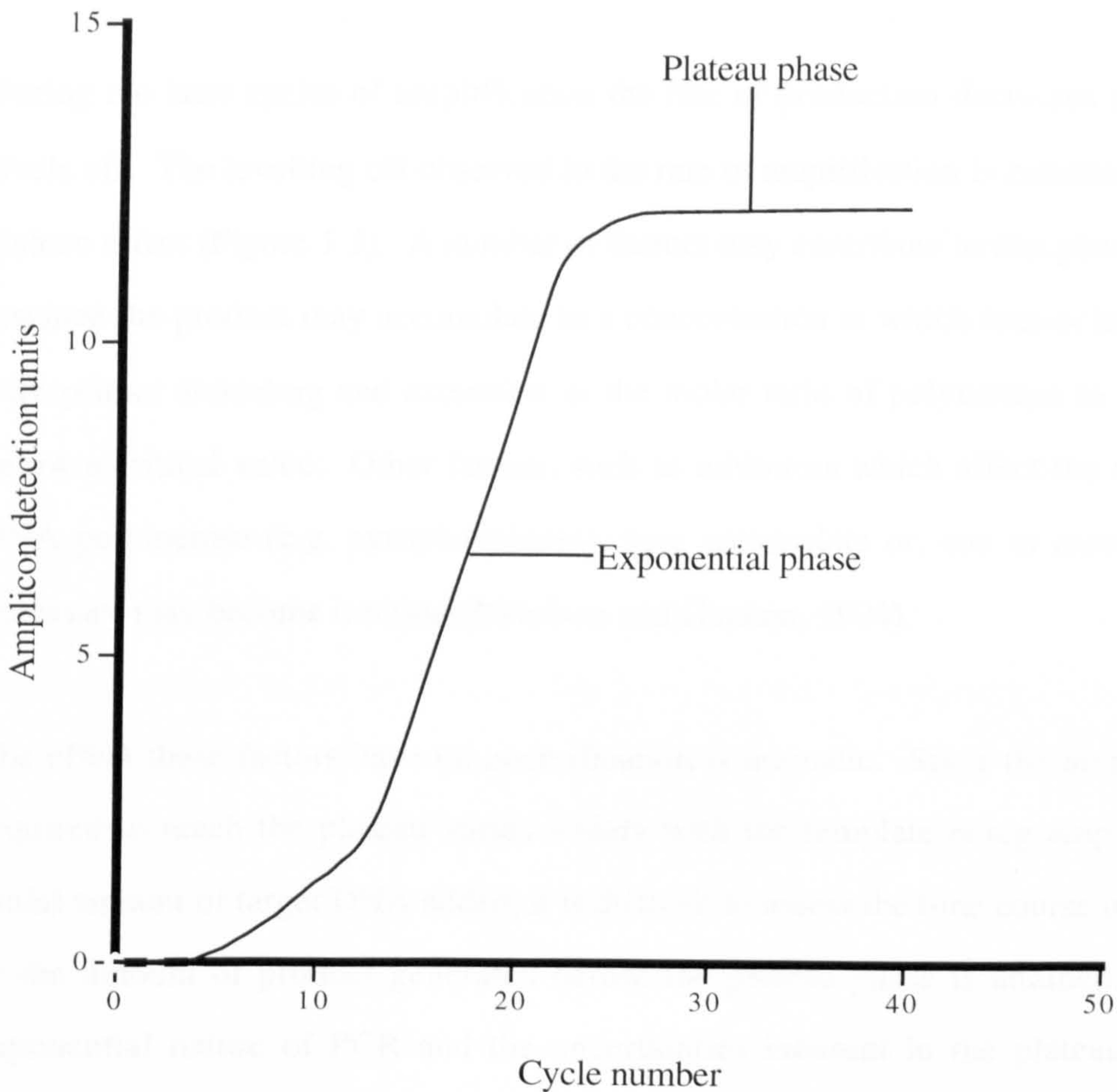


Figure 1.3 The exponential and plateau phases of PCR. During the exponential phase of PCR, no factor is limiting, and amplification products are generated at a steady rate. At some stage of the reaction the components become limiting. Consequently, the amplification efficiency drops and eventually becomes zero. This stage of the reaction is termed the plateau phase.

generated is seen to increase exponentially. This phase adheres to the mathematical description previously given in Equation 1 with the amplification efficiency of the target remaining consistent throughout this phase. The number of cycles before a reaction enters the exponential phase is dependent on the initial amount of starting material but usually occurs between cycles 10 and 20 of a PCR.

During the later cycles of amplification the rate of production decreases and eventually levels off. The levelling off observed in the rate of amplification is commonly termed the plateau effect (Figure 1.3). A number of factors may contribute to this phenomenon. For instance the product may accumulate to a concentration at which reassociation competes with primer annealing and extension or the molar ratio of polymerase to template falls below a critical value. Other factors, such as inhibitors which affect the activity of the DNA polymerase (e.g. pyrophosphates), may accumulate or, one or more components necessary may become limiting (Morrison and Gannon, 1994).

The effect these factors have on quantification is dramatic. Since the number of cycles required to reach the plateau varies widely with the template being amplified and the initial amount of target DNA added, it is difficult to assess the time course of the reaction or the amount of product generated before the plateau phase is attained. Due to the exponential nature of PCR and the uncertainties inherent in the plateau effect, it is difficult to carry out quantitative analysis using PCR. However, a number of reports have quantified targets using competitive PCR in the plateau phase (Becker-André and Hahlbrock, 1989; Clementi *et al.*, 1993; Cottrez *et al.*, 1994; Pannetier *et al.*, 1993). This is probably the exception rather than the rule. The main reason for using the plateau phase is that it does not require the additional work required to show that the product accumulates linearly up to and beyond the cycle number in which the signal is being measured. Instead, all measurements are taken at the same high cycle number. Since a considerable amount of work is initially required to establish that any measurements in the plateau phase are not subject to this kind of constraint, the advantage of plateau-phase

quantification is negated. It is evident from this that all quantification should be carried out in the exponential phase of amplification.

1.4.2 The formation of heteroduplexes

Although a mimic homologous to the target is required to ensure comparable amplification efficiencies, similarities in sequence composition between the target and mimic can also be a source of error. During the early cycles of PCR, the initial excess of primers ensures that once template DNA becomes denatured, it will anneal to primers rather than itself. Subsequent extension by *Taq* DNA polymerase generates homoduplexes (nucleic acid duplexes whose single strands are the precise complement of one another). As the polymerisation reaction proceeds through exponential amplification and approaches plateau, the ratio of template to primer increases dramatically with each cycle. This excess of product results in target and mimic strands re-annealing to one another creating heteroduplexes (non-specific annealing of dissociated strands to generate target-mimic and mimic-target duplexes). When mimics with insertions or deletions are used heteroduplex formation is reduced (Henley *et al.*, 1996; McCulloch *et al.*, 1995) however, with a mimic containing a point mutation, heteroduplex formation is more likely. In this instance, when the products are digested with restriction enzymes they are unable to digest heteroduplexes that contain recognition sites for the restriction enzyme on only one strand, any undigested products of the same size as the target would be mistakenly added to the balance of the undigested reaction product, causing error. A method involving the use of two restriction enzymes has been developed, in which both the target and mimic contain unique restriction sites (Hayward-Lester, 1996; McCulloch *et al.*, 1995). Any heteroduplexes generated will not be digested and therefore will remain uncut and separate from the target and mimic. In addition, other methods have been developed to monitor this problem and correct for heteroduplex formation (Becker-André and Hahlbrock, 1989; Hayward-Lester *et al.*, 1995; Hayward-Lester *et al.*, 1996; Henley *et al.*, 1996). The simplest is to restrict the number of amplification cycles in order to generate quantitative data prior to the formation of heteroduplexes (Schneeberger *et al.*, 1995).

However, as heteroduplex formation is dependent on the initial concentration of template, this approach is not a viable method of compensating for heteroduplex formation in routine quantitative PCR. Alternative preventative measures have been reported by Becker-André and Hahlbrock (1989), who demonstrate that an additional amplification cycle after dilution of the PCR products will eradicate heteroduplexes. However, this procedure reduces the total amount of PCR product generated, and further manipulations are required to restore sensitivity and enable the detection and subsequent quantification of amplicons, e.g. radioactive labelling, blotting and probe hybridisation.

1.5 *Legionella pneumophila* as a Model System for the Quantification of DNA using PCR

Legionella pneumophila is a ubiquitous water micro-organism and is a naturally occurring contaminant of many semi-contained water systems (Negron-Alvira *et al.*, 1988; States *et al.*, 1987; Yamamoto *et al.*, 1995). Under normal conditions the organism does not usually multiply significantly or pose a health threat. It is important that under these circumstances, samples are not identified as positive leading to expensive and inconvenient decontamination procedures. The reverse is also true with appropriate action needing to be taken where large numbers have arisen. Techniques currently used for the isolation of *Legionella* on culture media are often hampered by their long incubation period and fastidious growth requirements. Other problems associated with this technique include the inhibition of *Legionella* growth by other contaminating micro-organisms and the presence of viable, but unculturable *Legionellas* found in environmental samples (Hussong *et al.*, 1987). PCR has been used for the highly sensitive detection of *Legionella* species in environmental water samples (Maiwald *et al.*, 1994; Yamamoto *et al.*, 1995). Although it is important to be able to detect this organism successfully, for instance in order to ensure cooling towers are properly maintained and do not pose a health risk, it is also useful to know the number of cells present within a sample. This is important, as the Health and Safety Executive recommend that cooling towers be shut down and cleaned following the notification of a positive result (e.g. ≤ 10 cells) (HSE HS(G)70, 1991).

However, large-scale routine quantification of samples has been hindered by the amount of labour required for accurate quantification (Becker-André and Hahlbrock, 1989; Gilliland *et al.*, 1990). For this reason, *Legionella pneumophila* was selected as one of the model systems to develop a PCR based assay for the absolute quantification of DNA in a given sample. This in turn would allow the number of cells present in a sample to be ascertained and the level of contamination determined.

1.6 Aims and Rationale

The overall aim of this study was to determine the critical factors affecting the quantification of DNA using PCR and to use the findings to develop an assay for the absolute quantification of DNA in selected model systems. From the review of the literature, it is apparent that if the PCR is to become a routine technique outside of the research laboratory, then the presence of the specific PCR product must be confirmed independently by a non-radioactive assay which avoids gel electrophoresis. Furthermore, if the assay is to be used for quantification, then a "competitive PCR" maximises the information which may be derived from a given number of dilution's of template.

Recent advances in the real-time monitoring of amplification products using intercalating dyes has made kinetic quantification possible. The advantage of real-time acquisition of data over end-point detection is obvious. Fewer processing steps reduce the opportunity for error and contamination. Moreover, the time and effort required by these manipulations are substantially reduced. The aim of this work was to investigate whether it is possible to combine all of these features in a PCR to quantitate the absolute number of *Legionella* in environmental samples. Successful development of such an assay for *Legionella pneumophila* would demonstrate the feasibility of similar assays in routine environmental and clinical microbial assessment.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

The chapter which follows describes the various fundamental techniques employed for the preparation and manipulation of DNA. Several molecular biology manuals proved invaluable, including "Essential Molecular Biology: A Practical Approach" (Ed. Brown, T. A., 1991) and "Molecular Cloning: A Laboratory Manual", (Sambrook *et al.*, 1989).

2.1 Bacterial Strains

Escherchia coli cell lines XL1-Blue (XL1-B) and DH5- α were used for DNA manipulations. *E. coli* XL1-B and DH5- α was purchased from Stratagene Ltd. (Cambridge, UK). Their genotypes are described below in Table 2.1.

Table 2.1 Genotypes of *E. coli* cell lines.

<i>E. coli</i>	Genotype
XL1-B	<i>supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac⁻, F'[proAB⁺ lacI^q, lacZΔM15, tn10(tet^r)]</i>
DH5- α	<i>deoR, endA1, gyra96, hsdR17(r_k⁻m_k⁺) recA1, relA1, supE44, thi-1, Δ(lacZYA-ARGfv169), ϕ89ΔlacZΔM1</i>

Known counts of *Legionella pneumophila* in tap water were supplied by Dr Mike Tully, Senior Microbiologist at the Legionella Research Group, Porton Down (PHLS Centre for Applied Microbiology and Research, Salisbury, Wiltshire, UK.)

2.2 Microbiological Media and Methods

Freeze-dried powder media and/or media constituents were dissolved in double deionised water (ddH₂O) purified by a Milli Q system (Millipore UK. Ltd., Watford, Herts., UK.)

and sterilised by autoclaving at 121°C (15 psi) for 20 minutes on liquid cycle. The composition of all media used is given in Appendix I, together with any details of post autoclaving manipulations. All other chemicals were analytical grade, purchased from Sigma Chemical Company (Poole, Dorset, UK.). The antibiotics employed for specific strain selection (Appendix II) were purchased from Sigma Chemical Company (Poole, Dorset, UK.).

2.3 General Buffers and Chemicals

All general chemicals used were of analytical grade, purchased from either Sigma Chemical Company (Poole, Dorset, UK.), Fisher Scientific (UK.) Ltd., (Loughborough, Leics., UK.) or BDH (Merck Ltd., Poole, Dorset, UK.). Solutions were prepared in ddH₂O purified by a Milli Q system (Millipore, Herts. UK.) and were sterilised by autoclaving at 121°C (15 psi) for 20 minutes on liquid cycle. Recipes for buffers used are listed in Appendix III. All general chemicals and enzymes are listed in Appendix IV.

2.4 Growth, Maintenance and Storage of Bacteria

For small scale bacterial growth, a single bacterial colony was aseptically transferred from a stock plate into a bijoux tube (BDH Merck Ltd., Dorset, UK) containing LB media broth (3 ml) supplemented with the appropriate antibiotic (ampicillin, 100 µg ml⁻¹). All bacteria were cultivated aerobically at 37°C overnight. For large scale bacterial growth, a conical flask (1 L) containing nutrient medium (200 ml), supplemented with the appropriate antibiotic, was inoculated with an overnight culture (2 ml). For volumes greater than 25 ml, baffled flasks were used to maximise aeration. Bacterial cultures were stored at -70°C in glycerol (15% v/v).

2.5 Preparation of Genomic DNA

2.5.1 Removal of protein with phenol/chloroform

Removal of proteins from nucleic acid solutions was normally carried out by digestion with proteinase K, followed by phenol extraction. The sample (typically 1 ml) was

digested with proteinase K (100 $\mu\text{g ml}^{-1}$) for 1 hour at 55°C. Organic solvents such as phenol denature and precipitate protein contaminants in DNA samples. An equal volume of Tris-equilibrated phenol was added to the cell lysate and mixed by inversion. Tubes were centrifuged at 13,000 r.p.m. for 10 minutes (microcentrifuge tube scale-purification) (Alpha Laboratories, Hants., UK.) or 4,500 x g for 10 minutes (Falcon-scale purification) (Becton Dickinson Labware, New Jersey, USA.). Centrifugation causes the solution to partition into two separate phases, organic and aqueous. The aqueous phase (upper layer) contains dissolved nucleic acids while denatured proteins and cell debris collect as an insoluble white layer at the interface between the aqueous and organic layers. The aqueous phase was transferred to a fresh tube and an equal volume of phenol:chloroform (1:1 v/v) added. Tubes were mixed by inversion and centrifuged as previously described. If required, the interface was back-extracted by adding an equal volume of buffer and centrifuging as before to achieve maximum recovery. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was then added. The addition of chloroform removes any traces of dissolved phenol present in the aqueous layer, while isoamyl alcohol also reduces foaming, facilitating the separation of organic and aqueous phases. Samples were again mixed by inversion and centrifuged as before. The upper aqueous layer was removed and pipetted into a fresh tube, ready for nucleic acid extraction.

2.5.2 Concentration of DNA by alcohol precipitation

Alcohol precipitation was used to recover high molecular weight DNA from the aqueous phase. In the presence of moderate concentrations of monovalent cations, and at a temperature of -20°C or less, absolute ethanol efficiently precipitates nucleic acids. DNA samples were adjusted to 0.3 M with respect to sodium acetate (NaOAc) and total nucleic acids were precipitated with absolute ethanol (2 volumes) at -20°C for 2 hours, or at -70°C for 30 minutes. Precipitated DNA was recovered by centrifugation at 12,000 r.p.m. for 20 minutes at 4°C (microcentrifuge tube-scale extraction) or at 4,500 x g for 20 minutes at 4°C (Falcon-scale extraction). Traces of salt were removed by washing the DNA pellet

in 70% ethanol. The washed DNA pellet was subsequently dried at 65°C for 2-5 minutes and dissolved in the desired volume of buffer (T₈E, pH 8.0 at 20°C or ddH₂O).

2.5.3 Removal of RNA from DNA preparations

RNA contaminants were removed from DNA preparations by incubation with DNase-free RNase A (1 µg µl⁻¹) for 30 minutes at 37°C. DNase-free RNase was dissolved in ddH₂O (0.01 M, pH 5.2 at 20°C) to a final concentration of 10 mg ml⁻¹. The solution was heated to 100°C for 15 minutes and when cooled to room temperature, the pH was adjusted by adding Tris-HCl (1 M, pH 7.4 at 20°C). Stock RNase was stored in aliquots (1 ml) at -20°C.

2.5.4 Spectrophotometric quantification of DNA

The total amount of nucleic acid in a preparation was determined spectrophotometrically at 260 nm (GeneQuant, Pharmacia Biotech, St Albans, Herts., UK.). The quantity of DNA was calculated using the guide that an optical density (OD) of 1 corresponds to approximately 50 µg ml⁻¹ for double-stranded DNA. The purity of the DNA was determined using the ratio between A₂₆₀/A₂₈₀. Values less than 1.8 were indicative of protein contamination, therefore re-extraction with phenol:chloroform and ethanol precipitation was performed (Sections 2.5.1 and 2.5.2 respectively).

2.6 Polymerase Chain Reaction

All reagents used for PCR, with the exception of primers, were purchased from Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.). PCR reactions (typically 25-100 µl) were routinely performed in snap-lock microtubes (0.2 ml) (GeneAmp microtubes, Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.) containing a final concentration of Tris-HCl (10 mM, pH 8.3 at 20°C), KCl (50 mM), gelatin (0.001% w/v), dATP, dTTP, dCTP and dGTP (0.2 mM), *AmpliTaq* DNA polymerase (0.625 units),

0.8 μ M of each primer (Kings College, London, UK.), MgCl_2 (1.5 mM) and a variable amount of template DNA. For long PCR, a specified number of units of *Taq* Extender™ PCR additive (Stratagene, Cambridge, UK.) was added to the reaction mixture to increase the reliability and yield of amplification (1 unit of *Taq* Extender™ PCR additive per 1 unit of *AmpliTaq* DNA polymerase). The standard reaction buffer was replaced by *Taq* Extender™ reaction buffer [KCl (100 mM), Tris-HCl (200 mM, pH 8.8 at 20°C), MgSO_4 (20 mM), $(\text{NH}_4)_2\text{SO}_4$ (100 mM), Triton X-100 (1% v/v) and nuclease-free bovine serum albumin (BSA) (1 mg ml⁻¹)] according to the manufacturer's protocol. Each reaction, containing 100 ng of λ EMBL3 vector (Promega, Southampton, Hants., UK.), was adjusted to a final volume of 100 μ l with ddH₂O. The primers used for the generation of long PCR products are shown in Table 2.2. All long PCR primers, which were supplied by Perkin-Elmer Applied Biosystems (Warrington, Cheshire, UK.), were designed using basic guidelines suggested by Cheng *et al.*, (1994). PCR negative controls were routinely performed, using the reaction mixtures as described previously, but substituting ddH₂O for template DNA. Samples were amplified through 30 cycles in a programmable thermal cycler (GeneAmp®2400 PCR system, Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.). The amplification profiles employed were as follows:

Standard PCR: 1 cycle of denaturation at 95°C for 5 minutes was followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 3 minutes with a final cycle of 72°C for 7 minutes.

Long PCR: An initial cycle of denaturation at 94°C for 5 minutes was followed by 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for between 5-20 minutes, depending on target length, with a final extension step of 72°C for 10 minutes.

Profile for the amplification of degraded DNA: The first denaturation step of 95°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing at

50°C for 30 seconds and extension at 72°C for 2 minutes. The 30 cycles were followed by a final extension of 72°C for 7 minutes, with an indefinite hold at 4°C.

Table 2.2 PCR primers designed for the generation of long PCR products. The extension time used for the generation of each amplicon was determined by allowing 60 seconds of extension time for each 1 kb of target amplified.

Product size (kb)*	Positive strand primer	Negative strand primer	Extension time (minutes)
1	MBL 1007N	MBL 2007C	1
4	MBL 1007N	MBL 5007C	4
9	MBL 1007N	MBL 10,007C	9
14	MBL 1007N	MBL 15,007C	14
19	MBL 1007N	MBL 20,007C	19

2.6.1 PCR product purification

The presence of non-specific amplification products, residual oligonucleotide primers, primer dimers, unincorporated nucleotides and buffer reagents can adversely affect subsequent processing of the PCR product. The PCR product was purified from both solution and agarose matrices as follows:

From agarose: When required for further manipulation, PCR products were purified following agarose gel electrophoresis using the Mermaid Kit (Bio 101, Middlesex, UK.). This is a resin-based purification procedure which utilises silica particles ("glass fog") for the adsorption of low molecular weight DNA. Briefly, the desired DNA "band" was excised from the agarose gel and dissolved by vigorous vortexing in sodium perchlorate solution (3 volumes) containing 8 µl "glass fog" per µg of DNA. The glass fog was washed several times with an ethanol-containing buffer to remove agarose impurities and the DNA eluted twice at 50°C with an appropriate volume of ddH₂O. Percentage recovery was evaluated by horizontal gel electrophoresis of typically 1/10 of the eluted

DNA, and DNA concentration was determined by UV absorbance spectroscopy (as described in Section 2.5.4).

From solution: When required, PCR products were purified from solution using Microcon-100 ultrafiltration units (Millipore, Watford, Herts., UK.). This technique is based on the ultrafiltration of a sample through a molecular weight cut-off filter membrane. Before use, the unit was pre-rinsed with ddH₂O to remove traces of glycerin from the membrane. The PCR reaction (25-100 µl) was diluted with ddH₂O (500 µl) and placed in the sample reservoir of the microconcentrator. The device was centrifuged at 500 x g for 30-40 minutes to drive buffer components and low molecular weight solutes through the membrane. All DNA fragments above the membrane's molecular weight cut-off were retained in the sample reservoir. The volume of the retentate was diluted with ddH₂O (500 µl) and the entire procedure repeated twice. The final retentate was recovered by placing the microconcentrator unit upside down in an eppendorf tube (0.5 ml) and centrifuging briefly. The successful removal of primers was verified by horizontal gel electrophoresis and DNA concentration was determined by UV absorbance spectrophotometry (as described in Section 2.5.4).

2.7 DNA Restriction Digestion

Type II restriction endonucleases recognise specific nucleotide sequences in double-stranded DNA and cleave the duplex at these sites. All restriction enzymes used were supplied by Pharmacia Biotech (St Albans, Herts., UK.). Typically, DNA (0.2-1 µg) was digested in a final reaction volume of 20 µl, containing 2 µl One-Phor-All restriction enzyme buffer (10 x) and 1 µl of the appropriate restriction enzyme. Digestion was performed in a heated water bath at 37°C for 1-2 hours and digestion products were analysed by agarose gel electrophoresis as described in Section 2.8.

2.8 Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis is the standard method for separating DNA molecules and was used to verify the integrity and purity of DNA preparations, to analyse PCR products and to resolve DNA fragments following restriction enzyme digestion. DNA molecules are negatively charged, and under the influence of an electric current, are resolved according to their size by migration through an agarose gel matrix. The amount of agarose in the gel determines the size range of DNA fragments that can be separated: smaller DNA molecules migrate faster while the movement of larger molecules is retarded by the nature of the gel matrix.

Agarose was melted in TBE buffer (1 x) and allowed to cool to approximately 50°C, prior to the addition of ethidium bromide (0.5 µg ml⁻¹). The gel mixture was then poured into a gel casting tray and left to set at room temperature for approximately 30 minutes. DNA samples (typically 10 µl) were applied to separate wells in the gel in a loading buffer that contained Bromophenol Blue (0.05% w/v) and glycerol (5% v/v). Bromophenol Blue is a tracking dye and is used to follow the migration of the DNA front through the gel. Glycerol increases the sample solution density ensuring the sample is retained in the well once loaded. Electrophoresis was performed in TBE buffer (1 x), containing ethidium bromide (0.5 µg ml⁻¹), at between 80-150 volts (V), depending on the gel size (Table 2.3).

Table 2.3 Conditions employed for agarose gel electrophoresis.

% agarose	Gel (cm)	Voltage (V)	Time (min)
0.7-1.0	10	80-100	45-60
1.5-2.0	20	150	60

The incorporation of ethidium bromide in the gel and running buffer allowed the visualisation of DNA fragments under UV illumination (UV Transilluminator, UVP Inc.,

California, USA). Photographs were taken through an orange filter using a Polaroid MP-4 Land Camera with Polaroid 667 black and white film (Herts., UK.). The size of DNA molecules were determined by comparing their migration with DNA fragments of a known size, e.g. 1 kb DNA ladder (Gibco BR, Paisley, Scotland, UK.) or a 100 bp ladder (Pharmacia Biotech, St Albans, Herts., UK.). In general, 1% agarose gels were used for visualising high molecular weight genomic DNA and plasmids, while 1.75% gels were used to resolve PCR products less than 1 kb in size.

2.9 Southern Blotting

Following electrophoresis, DNA was transferred onto nylon membranes essentially as described by Southern (1975). All manipulations were performed at room temperature. The gel was immersed in alkali denaturing solution and shaken for 20 minutes to break the DNA into smaller fragments. This process was omitted if small DNA fragments such as PCR products were to be transferred. The gel was then placed in neutralising solution and agitated for a minimum of 2 hours using an orbital shaker (Stovall Life Science Inc., North Carolina, USA.).

To prepare the gel for Southern blotting a deep plastic tray was filled with SSC (6 x) and a perspex sheet placed on top of the tray to act as a support for the gel. A piece of 3MM chromatography paper was used to cover the perspex sheet and the ends of the paper allowed to dip into the SSC (6 x) to form wicks. The gel was then inverted onto the 3MM paper, dampened with SSC (6 x) and a nylon membrane, cut to size, placed onto the gel carefully to avoid the formation of air bubbles. Two pieces of 3MM paper, cut to the same size of the gel, were placed onto the nylon membrane and dampened with SSC (6 x). Plastic gel guards were then placed around the edge of the gel to ensure the SSC (6 x) was drawn up through the gel by capillary action. Two absorbent wipes were then placed on top of the 3MM paper followed by a stack of paper towels, a perspex sheet and a stabilising weight. The transferred nucleic acids were bound to the membrane by

exposure to ultraviolet radiation for 30 seconds using a Stratalinker 2400 (Stratagene, Cambridge, UK.).

2.10 Radiolabelling of DNA

2.10.1 5'-end labelling of oligonucleotide probes using polynucleotide kinase

To ensure the efficiency of the reaction equimolar quantities of [γ - ^{32}P] ATP and oligonucleotide probe were used for all probes prepared by this method. 1 μl of a oligonucleotide probe (4 μM) was added to a screw cap tube (2.0 ml) containing ddH₂O (15 μl) and heated in a waterbath to 65°C for 1 minute. The reaction was then placed on ice and cooled for 30 seconds. 2 μl 10 x kinase buffer, 0.5 μl spermidine (100 mM) and 0.5 μl polynucleotide kinase (PNK) (5 units μl^{-1}) were added to the reaction tube, mixed and incubated at 37°C for 15 minutes. The reaction mixture was then heated to 65°C for 1 minute and allowed to cool on ice for 30 seconds. Following the further addition of 1 μl β -mercaptoethanol (35 mM) and 1 μl PNK (5 units μl^{-1}) the reaction mixture was again incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 5 μl EDTA (500 mM).

2.10.2 Primed DNA labelling

The preparation of oligonucleotide probes by this method was adopted from a protocol by Salutz and Jost (1989).

Briefly, an annealing mix for the oligonucleotide probe was prepared consisting of 10 μl of the normal strand primer, 30 bases in length (5 pmole), 4 μl of the complementary primer, 10 bases in length (5 pmole) and 2.5 μl 10 x oligonucleotide buffer. The annealing mix was incubated at 75°C for 2 minutes then left at room temperature for 20 minutes to cool and then placed ice for 10 minutes. 1 μl each of dGTP, dTTP, and dCTP (10 mM) was added to the annealing mix. The annealing mix (2 μl) was added to a microcentrifuge tube (0.5 ml) containing 1.5 μl [γ - ^{32}P] dATP (555 kbq, 111 Tbq mmol⁻¹) and 0.5 μl Klenow fragment (5 units μl^{-1}) and incubated at room temperature for 1 hour.

The reaction was adjusted to a final volume of 50 μ l with ddH₂O.

2.10.3 Precipitation of oligonucleotide probes with trichloroacetic acid (TCA) to calculate the percentage incorporation of radiolabel

Radiolabelled oligonucleotide probes (2 μ l) were added to aliquots of ddH₂O (98 μ l) and 50 μ l of the aliquot spotted onto 2 glass fibre discs (Whatman GF/C 2.4 cm diameter, Watford, Herts., UK.). 1 disc was washed 3 x 5 minutes in ice-cold TCA (5% w/v) to remove unincorporated radiolabel. Both washed and unwashed filters were dried at 80°C for 1 hour. The discs were added to separate scintillation vials and scintillation fluid (6 ml) (β -fluor, Applied Diagnostics Ltd., Bucks., UK.) added. The disintegration's were measured using a 1900TR liquid scintillation analyser (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.). The amount of radioactivity on the unwashed filter was compared with the amount on the washed filter to calculate the proportion of unincorporated nucleotide precursor.

2.11 Hybridisation of Southern Blots

2.11.1 Preparation of nylon membranes for hybridisation

Each nylon filter (HybondN, Amersham International plc., Little Chalfont, Bucks., UK.) was sandwiched between 2 nylon membranes (Amersham International plc., Bucks., UK.) and soaked in a plastic tray containing SSC (2 x), taking care to avoid the formation of air bubbles. The sandwiched nylon filter was then rolled up to fit into a glass hybridisation bottle. 10 ml SSC (2 x) was added to the bottle and the bottle rolled to allow the membrane sandwich to unwind itself onto the wall of the bottle. The SSC (2 x) was subsequently discarded, the bottle drained of any remaining solution, and hybridisation buffer (10 ml) added to the bottle. The nylon filters were then placed in prehybridisation buffer at 42°C for at least 2 hours. To the prehybridisation buffer approximately 3×10^5 c.p.m. ml⁻¹ of ³²P radiolabeled oligonucleotide was added and the membranes incubated overnight at 42°C in a Mini-10 rotisserie hybridisation oven (Hybaid, UK.). Unbound probe was subsequently removed with 4 x 10 minute washes using high stringency wash

buffer and the membrane prepared for exposure to x-ray film.

2.11.2 Autoradiography

The membrane was transferred to an EC x-ray film cassette (Fuji, Japan) containing a Mach 2 intensifying screen (Fuji, Japan) and placed onto RX-ray film (20 cm x 40 cm (Fuji, Japan)). The cassette was typically stored at -70°C overnight prior to development of the autoradiographic image. All autoradiographs were subsequently re-exposed to x-ray film for a further 7 days to allow the development of very weak hybridised signals.

2.12 Cloning of PCR Products

2.12.1 Preparation of competent cells

A baffled flask containing LB media broth (40 ml) was inoculated with an overnight *E. coli* XL1-B culture (2 ml), and grown aerobically at 37°C for 2-3 hours. Growth was monitored spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, St Albans, Herts., UK.) and cells were harvested at mid-logarithmic phase ($A_{600\text{nm}} = 0.3\text{-}0.5$ units) by centrifugation at 3,000 x g (Jouan CR422 centrifuge, Jouan, France) for 10 minutes at 4°C. The resultant pellet was gently resuspended in 0.5 volumes (20 ml) of ice-cold sterile CaCl_2 (50 mM), and placed on ice for 40 minutes. The cells were harvested as previously, resuspended in 0.1 volumes (4 ml) of ice-cold CaCl_2 (50 mM) and incubated on ice for 2 hours before use.

2.12.2 Transformation of competent cells

1-10 ng of plasmid DNA was added to aliquots of ice-cold competent cells (100 µl). For a negative control, a reaction containing no plasmid DNA was used. Following a 40 minute incubation on ice, cells were heat pulsed in a 42°C waterbath for 45 seconds, and then placed immediately on ice for 2 minutes. Preheated (42°C) SOC media (0.9 ml) was added, and the transformation mixture incubated at 37°C for 1 hour with constant agitation. Bacteria were harvested by centrifugation at 3,000 x g for 10 minutes, and the cells resuspended in SOC media (200 µl). Transformants were selected by plating onto

LB broth agar supplemented with ampicillin (0.1 mg ml^{-1}). Plates were incubated at 37°C overnight and individual colonies were verified by DNA extraction (miniprep) and PCR screening (Sections 2.13.2 and 2.6 respectively).

2.12.3 Large scale cloning of PCR products

Large numbers of PCR products were cloned using the TA cloning kit (Invitrogen Corporation, Netherlands). PCR products were diluted to approximately 10 ng ml^{-1} and ligated directly into the pCRTM 2.1 vector (138 pg) using T4 ligase according to the manufacturer's protocol. Ligation reactions were incubated overnight at 14°C and the ligated products ($1 \mu\text{l}$) added to competent *E. coli* DH5- α cells. The suspension was incubated on ice for 30 minutes, followed by 1 minute at 42°C and 1 hour at 37°C . Transformed cells were selected on ampicillin plates containing IPTG (100 mM) and X-Gal (2% w/v).

After overnight incubation at 37°C , recombinant cells containing an insert, grew as white colonies owing to insertional inactivation of the β -galactosidase gene whereas cells containing plasmid with no insert retained β -galactosidase activity and were blue. Cells with no plasmids failed to grow. White colonies were selected and grown to stationary phase in LB media broth (3 ml) supplemented with ampicillin ($100 \mu\text{g ml}^{-1}$).

2.13 Isolation of Plasmid DNA

2.13.1 Large scale plasmid DNA preparations

Wizard Maxiprep kits were used to prepare and purify large scale plasmid DNA preparations. These kits were used according to the manufacturer's instructions.

2.13.2 Small scale plasmid DNA preparations

Wizard Miniprep kits were used according to the manufacturer's instructions to prepare and purify small scale plasmid DNA preparations.

2.14 Preparation of Constructs for the Model System

To establish a consistent set of targets and mimics for the model system, a set of randomly picked clones ranging in size from 300 bp to 900 bp were derived directly from *Bacillus subtilis* or *B. cereus* and *Legionella pneumophila* by PCR using Leg 1 and Leg 2 primers (Starnbach *et al.*, 1989) (Table 2.4). These PCR fragments were ligated into the pCR 2.1 plasmid according to the supplier's instructions (Invitrogen, Netherlands). The ligation mixes were propagated in *E. coli* DH5- α (Stratagene, Cambridge, UK). The resulting clones were screened by PCR using both Leg and M13 primers to determine the presence and size of the insert. Standard stocks of each mimic were prepared using Wizard Maxiprep kits (Section 2.13.1) and the concentration of these targets determined by UV-absorption spectroscopy (Section 2.5.4). Further targets and mimics were constructed by amplifying sequence regions of the pGEM luc Basic 2 plasmid (Table 2.5) using primers designed to contain the first 20 nucleotides of the target sequence, flanked by the Leg primer recognition sites. Sections of the oligonucleotides containing the Leg primer sequences are underlined (Table 2.6).

Table 2.4 Constructs prepared for the model system using Leg primers.

Name	Source	GC content	Tm (°C)*	Construct size (bp)
SM1	<i>L. pneumophila</i>	53%	81.2	900
SM7	<i>B. subtilis</i>	39%	75.3	588
SM31	<i>B. subtilis</i>	47%	79.7	455
SM20	<i>B. cereus</i>	44%	79.1	304

Primer sequences: Leg 1; 5'-GTCATGAGGAATCTCGCTG-3'; Leg 2, 5'-CTGGCTTCTTCCAGCTTCA-3'.

*Tm calculated in PCR buffer (1 x).

2.15 Preparation of Degraded DNA

High molecular weight calf Thymus DNA (105 $\mu\text{g ml}^{-1}$) and fragmented calf thymus

Table 2.5 Targets and mimics generated using the pGEM luc Basic 2 plasmid as a template.

Name	GC content	Tm (°C)*	Construct size (bp)
Targets			
SM2	45%	77.8	900
SM8	45%	77.5	600
SM32	45%	77.3	450
Mimics			
SM21	40%	76.6	304
SM22	53%	81.5	304
SM23	52%	81.2	304
SM24	49%	80	304

*Tm calculated in PCR buffer (1 x).

Table 2.6 Primer sequences used for the construction of mimics using the pGEM luc Basic 2 plasmid.

Name	Primer sequence 5'-3'	Construct generated
Leg1L 570N	<u>GTCATGAGGAATCTCGCTGCTTTTGGCG</u> AAGAATGAAAA	304 bp (40% GC)
Leg2L 816C	<u>CTGGCTTCTTCCAGCTTCACCCTTCCGCA</u> TAGAACTGCC	
Leg1L 60N	<u>GTCATGAGGAATCTCGCTGGCGTCGAGT</u> TTTCCGGTAAG	304 bp (53% GC)
Leg2L 306C	<u>CCTGGCTTCTTCCAGCTTCAATACAAAG</u> GATATCAGGTGG	
Leg49%GCN	<u>GTCATGAGGAATCTCGCTGACAACGGC</u> GCGGGAAGTTCA	304 bp (49% GC)
Leg49%GCC	<u>CTGGCTTCTTCCAGCTTCAGATTATGTC</u> CGGTTATGTAA	
Leg53%GCN	<u>GTCATGAGGAATCTCGCTGCCTTTCGGT</u> ACTTCGTCCAC	304 bp (53% GC)
Leg53%GCC	<u>CTGGCTTCTTCCAGCTTCACCGCTTGAA</u> GTCTTTAATTA	
Leg31L 480N	<u>GTCATGAGGAATCTCGCTGCATAACCGG</u> ACATAATCATA	450 bp (45% GC)
Leg31L 861C	<u>CTGGCTTCTTCCAGCTTCAATGTATAGAT</u> TTGAAGAAGA	
Leg7L 500N	<u>GTCATGAGGAATCTCGCTGTTAACGCCCA</u> GCGTTTTCCC	600 bp (45% GC)
Leg7L 1061C	<u>CTGGCTTCTTCCAGCTTCACCTCTGGATC</u> TACTGGGTTA	
Leg1 245N	<u>GTCATGAGGAATCTCGCTGGCCACGCCC</u> GCGTCGAAGAT	900 bp (45% GC)
Leg1 1106C	<u>CTGGCTTCTTCCAGCTTCACAGAGTCCTT</u> TGATCGTGAC	

DNA (12.5 $\mu\text{g ml}^{-1}$) were dissolved in sterile distilled water and the fragmented calf thymus DNA degraded further by sonication using a MSE Soniprep 150 (Fisher Scientific, Loughborough, Leics., UK.) at an amplitude of 4 μm for 0-120 seconds. Beef samples were also subjected to microwaving for selected periods of time using a Phillips M734 microwave (Phillips, London, UK.).

2.15.1 3'-end labelling of microwaved beef samples and 5'-degenerate competitive primer analogues with digoxigenin-11-ddUTP

DNA was extracted from microwaved beef samples using the method described by Sambrook *et al.*, (1989). Degraded calf thymus DNA and the 5'-degenerate competitive primer analogues were 3'-end labelled with digoxigenin-11-ddUTP using the DIG Oligonucleotide 3'-end labelling kit (Boehringer Mannheim, Lewes, UK.) according to the manufacturer's instructions. Approximately 100 pg of DNA was used for 3'-end labelling with terminal transferase to ensure completeness.

2.16 Quantification of PCR Products

2.16.1 Image intensity analysis of agarose gels

Analysis was done by electrophoresis of the PCR product (10 μl) on agarose gels (1.75%) containing TBE buffer (1 x) and ethidium bromide (0.5 $\mu\text{g ml}^{-1}$). The gels were viewed by UV illumination and photographed (Section 2.8). Scans of the gel were taken using a Sharp JX-610 flatbed scanner (Sharp, Middlesex, UK.). The image was inverted to resemble an x-ray film prior to quantitative analysis using a Bioimage analyser (Bioimage, Cheshire, UK). Due to differences in the size of the mimics amplified, the disparity between the percentage of ethidium bromide incorporated (relative to the size of the mimic) had to be taken into account. The band image intensities obtained were corrected for size using Equation 2 (McCulloch *et al.*, 1995):

Corrected band intensity = image intensity of DNA target / (target bp length / mimic bp length)

Equation 2: where bp = basepair.

Following analysis, the log value of the amount of target generated / amount of mimic product generated was plotted against the log of the initial number of target molecules added, to determine the initial number of starting molecules present in the sample.

2.16.2 Capillary electrophoresis (CE)

All experiments were carried out using the HP^{3D} Capillary Electrophoresis system (Hewlett-Packard, UK.) employing a CEP (polymer) coated capillary (48.5 cm x 75 µm i.d.). The temperature was maintained at a constant 25°C. Prior to CE analysis the capillary was flushed with DNA buffer for 10 minutes. PCR samples were electrokinetically injected at -5 kV for 35 seconds and pGEM DNA standards (1 mg ml⁻¹) injected at -4 kV for 5 seconds. These standards were used to calibrate the capillary and consisted of 15 fragments ranging in size from 36 bp to 2645 bp. An applied voltage of 16.5 kV (340 v cm⁻¹), with a voltage ramp of 0.3 minutes, was used for the analysis of PCR products. The on-line monitoring of samples was performed at a Diode Array Detection (DAD) wavelength setting of 254 nm (bandwidth, 10 nm) with a reference wavelength of 380 nm (bandwidth, 80 nm). Two concentrations of a sieving polymer buffer were prepared according to the manufacturer's instructions (Hewlett-Packard). A DNA buffer was prepared using hydroxyethyl cellulose (HEC) (1.5%) dissolved in electrolyte buffer (89 mM Tris, 332 mM Borate, 2 mM EDTA, pH 7.4) for the separation of short PCR products and another HEC solution (0.75%) for the analysis of larger products. Following CE, the ratio of the integrated peak areas was used to calculate the absolute amounts of target and mimic products in each tube. The area of each standard peak calculated in Section 4.2 represents the mean of three electropherograms.

2.16.3 High performance liquid chromatography (HPLC)

Samples were quantified by HPLC using the methodology described by Katz and Dong (1990). The chromatographic system (Knauer, London, UK.) consisted of a solvent

delivery system, a UV detector and an autosampler with a 20 μ l loop. Data was collected using a Shimadzu integrator (Kyoto, Japan). Samples were injected automatically every 15 minutes onto an ion exchange DEAE-NPR column (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.), 35 x 4.6 mm i.d., packed with 2.5 μ m particles. DNA was separated by employment of a gradient programme for the binary mobile phase as follows: reservoir A contained NaCl (1 M) and Tris-HCl (25 mM, pH 9.0 at 20°C); reservoir B contained Tris-HCl (25 mM, pH 9.0 at 20°C); the mobile phase was linearly changed from 70% to 60% B in 10 seconds, then from 60% to 48% B in 3 minutes and from 48% to 42% B in 5 minutes; 100% A was used for a 1 minute clean up before re-equilibration (5 minutes, 70% B). UV absorbency was measured at 254 nm. Under these conditions, all fragments were completely separated and the reproducibility of retention times and peak areas were as described by Katz and Dong (1990). The area of each standard peak calculated in Section 4.2 represents the mean of three chromatograms.

2.16.4 Continuous fluorescence monitoring of PCR products

All PCR amplifications were performed in Tris (50 mM, pH 8.5 at 25°C), $MgCl_2$ (3 mM), BSA (500 μ g ml⁻¹), 0.5 mM of each dNTP (Pharmacia Biotech, St Albans, Herts., UK.) and 0.2 units *Taq* DNA polymerase or *Amplitaq* Gold (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.). Primers (Kings College, London, UK.) were used at a final concentration of 0.5 μ M. SYBR Green ITM (Molecular Probes Europe BV, Netherlands) was also included in the reaction mix at a 1:10,000 dilution. Reactions were carried out in a total volume of 5 μ l and amplified using a LightCyclerTM (Idaho Technology Inc., Idaho Falls, USA.). PCR reagents were pipetted into the open plastic reservoir of composite plastic/glass capillary reaction tubes (Bio/Gene Ltd., Cambridge, UK.). The thermocycling profile consisted of denaturation at 94°C for 0 seconds, annealing at 50°C for 0 seconds and extension at 72°C for 30 seconds, repeated for 45 cycles. The ramp rate used for the denaturation and annealing steps was the same at 20°C s⁻¹, while a temperature transition rate of 3°C s⁻¹ was chosen for the extension reaction. If *Amplitaq* Gold was used in reactions an additional denaturation step of 12 minutes was included to

activate the enzyme prior to cycling. To allow the acquisition of fluorescence data for melting curve analysis, the amplification profile was immediately followed by a linear temperature transition from 72°C to 94°C at 0.1°C. All data were monitored continuously using a Labview user interface (National Instruments, Austin, USA.).

2.16.4.1 Determination of amplification efficiency using the Lightcycler™

PCR amplification was monitored in real time for 45 cycles. The cycle-by-cycle increase in fluorescence during the log-linear phase of amplification was monitored for the targets and mimics and the background fluorescence subtracted from the actual fluorescence. The result was plotted as a log-plot, extracting 0 or negative numbers. The amplification efficiencies of the targets and mimics were calculated from the linear slope of the graph using Equation 3:

$$E = (\text{inverse log of the slope of the graph}) - 1$$

Equation 3: where E = amplification efficiency.

The value for the slope of the graph was obtained by linear regression analysis of the log-plot. Melting curve analysis was used to confirm amplification of the intended product by its melting temperature.

2.16.5 Calculation of copy number

To calculate the number of copies of a target in a given amount of purified DNA the following formula was used.

$$\text{Number molecules (copies)} = \text{number of moles} \times 6.023 \times 10^{23}$$

Equation 4: where the molecular weight of a DNA molecule (MW) = number of nucleotides x 660 and the number of moles = weight (g)/MW

For the calculation of target copy number a genome size of 3.9 MB for *Legionella pneumophila* (Bender *et al.*, 1990) and 4.8 kb for the linearised plasmid (containing the 900 bp target sequence) were used.

Tap water samples containing known quantities of *Legionella pneumophila* were provided by Dr Mike Tulley (Centre for Applied Microbiological Research, Porton Down, Salisbury). Cell numbers were initially determined by plate count and then diluted accordingly to produce tap water standards of known cell number.

2.17 Sequencing

All PCR products from Section 2.6 were sequenced on an ABI 377 fluorescent sequencer using the reagents and protocols recommended by the manufacturer (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.). PCR products were cleaned using Wizard minicolumns (Promega, Southampton, Hants., UK.) before cycle sequencing was carried out using the Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, UK.) according to the manufacturer's instructions. DyeDeoxy terminators were removed from the cycle sequencing reaction using Centri-sep columns (Princetons Separations, Utah, USA.) before being loaded onto the sequencing gel (34 cm).

CHAPTER 3

DETERMINATION OF CRITICAL FACTORS INFLUENCING THE QUANTIFICATION OF DNA IN A MODEL SYSTEM BY COMPETITIVE PCR

3. DETERMINATION OF CRITICAL FACTORS INFLUENCING THE QUANTIFICATION OF DNA IN A MODEL SYSTEM BY COMPETITIVE PCR

3.1 Introduction

A major prerequisite for the accurate performance of a quantitative competitive PCR-based assay is that the mimic should amplify with an efficiency equal to that of the target. It is therefore important to determine any potential differences in amplification efficiency when developing such an assay by monitoring the ratio of target to mimic during amplification. These differences may become particularly significant during the exponential phase of amplification since small differences in the amplification efficiencies of either the target or mimic templates can translate into significant differences in final product yield over 30 or 40 PCR cycles. It has been suggested that the amplification efficiencies of both the target and mimic should be monitored at all stages of the reaction (Becker-André and Hahlbrock, 1989).

Differences in amplification efficiencies can arise when the length or composition of the mimic sequence differs from that of the target sequence, even if only by a few bases (McCulloch *et al.*, 1995; Henley *et al.*, 1996; Weissensteiner and Lanchbury, 1996). Although it is important to minimise any size differences between the target and mimic to ensure equal amplification efficiencies (Menzo *et al.*, 1992), any small differences in amplification efficiency can usually be compensated for by using a correction factor (McCulloch *et al.*, 1995).

Sequence similarities between the target and mimic products can lead to the formation of heteroduplexes as the PCR approaches the plateau phase of amplification (Henley *et al.*, 1996). Although such sequence similarity is essential for the amplification efficiency of

both the target and mimic products to be equal, it can interfere with the subsequent quantification of reaction products (Becker-André and Hahlbrock, 1989; Piatak *et al.*, 1993; Schneeberger *et al.*, 1995). Since the initial concentration of a target is calculated from the ratio of the final reaction products, the formation of heteroduplexes between a target and mimic will subtract equimolarly from both homoduplex products. Consequently, great care must be taken to avoid the formation of heteroduplexes by carefully designing and testing the mimic in competitive reactions (Apostolakos *et al.*, 1993; de Kant *et al.*, 1994; Zimmermann and Mannhalter, 1996).

Although differences in the sequence composition and size of a target are known to affect amplification efficiency (Zimmermann and Mannhalter, 1996), more recent work suggests that the amplification efficiency of two co-amplified targets can also be affected by differences in the thermal stability of their internal sequences (Weissensteiner and Lanchbury, 1995). This can cause the preferential amplification of one product over another under suboptimal PCR conditions, leading to the assignment of false negative results.

The aim of this investigation was therefore to determine the critical factors affecting the validity of competitive PCR for quantification purposes. To this aim, the influence of both target sequence composition and length on the amplification efficiency of different PCR targets in a competitive reaction were examined.

3.2 Results

3.2.1 The effect of target length on competitive PCR

In order to determine the effect of target length on competitive PCR, three targets were constructed with a GC content of 45% and sizes of 450 bp, 600 bp and 900 bp (Section 2.14). These targets were constructed with the same % GC content to allow them to amplify independently of any sequence specific effects. A dilution series of each target was amplified in the presence of a number of mimics, each of which were 304 bp in length and possessed different GC contents. Figure 3.1 shows a typical gel of the results obtained whilst Table 3.1 shows the initial ratios of target and mimic required to give a final target:mimic ratio of 1:1. Since the amount of target and mimic added to the competitive reactions was known, the ratio required to give an equal amount of both the target and mimic product could be determined. The point at which equimolar quantities of both the target and mimic PCR products were generated was determined by Image Intensity analysis (Section 2.16.1).

Table 3.1 The effect of sequence length on the initial starting ratio of molecules required to produce an equal amount of both target and mimic product.

% GC (304bp mimic)	900bp*:304bp	600bp*:304bp	450bp*:304bp
40%	4:1	0.7:1	0.21:1
44%	0.4:1	0.175:1	0.066:1
52%	Not obtained	Not obtained	Not obtained

*All of the DNA targets used in the competitive PCRs had GC content of 45%.

The initial starting ratios of molecules required to produce an equal amount of both target and mimic product decreased with increasing target length and GC content of the mimics suggesting a reduction in the amplification efficiencies of these mimics.

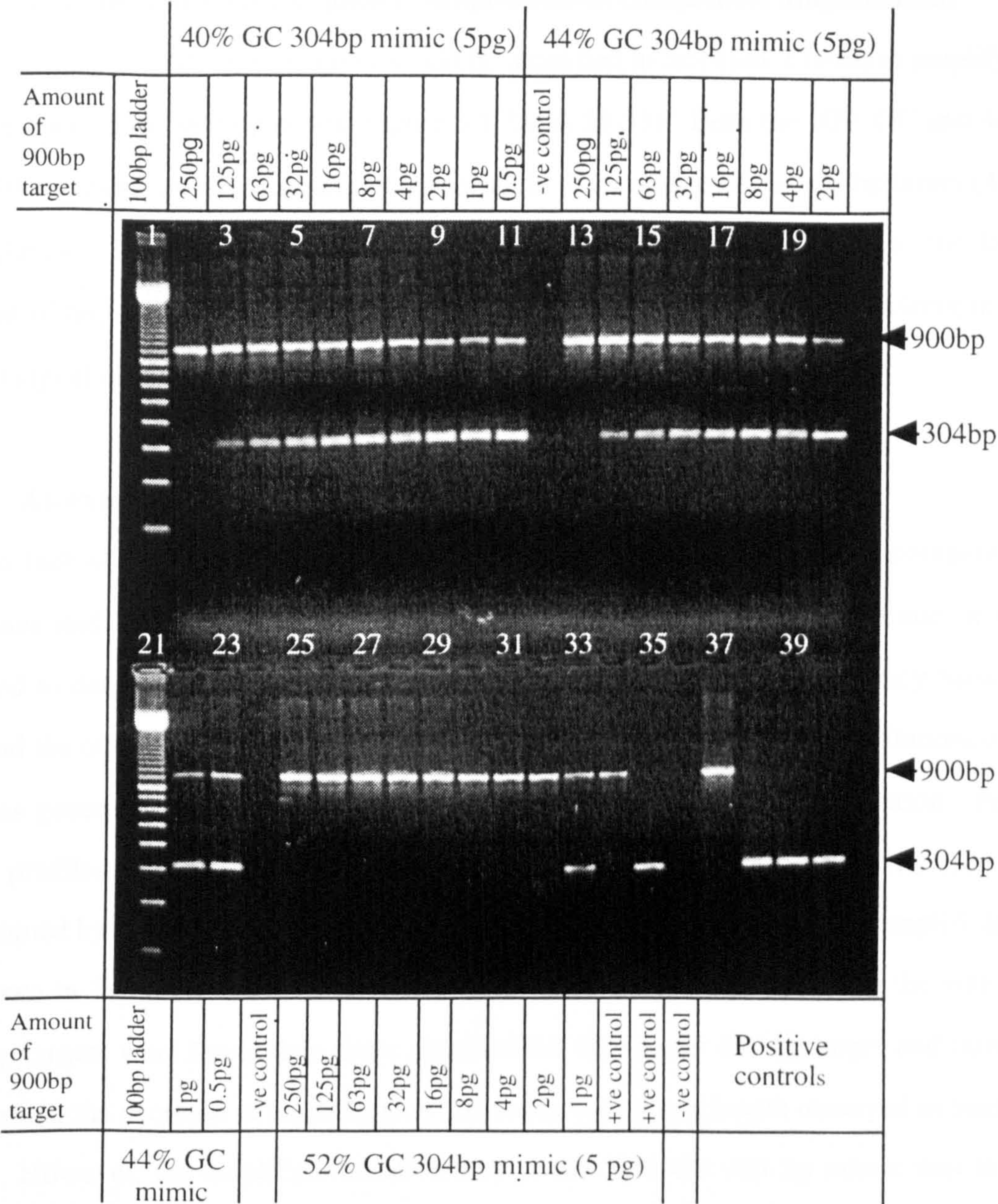


Figure 3.1 Competitive PCR products generated using a 900 bp DNA target and 304 bp mimics of varying % GC contents. Lane 1, 100 bp ladder; lanes 2-11, dilution series of a 900 bp target amplified in the presence of 5 pg 40 % GC 304 bp mimic; lane 12, negative control; lanes 13-23, dilution series of a 900 bp target amplified in the presence of 5 pg 44 % GC 304 bp mimic; lane 21, 100 bp ladder; lane 24, negative control; lanes 25-33, dilution series of a 900 bp target amplified in the presence of 5 pg 52 % GC 304 bp mimic; lane 34, 900 bp target (5 pg); lane 35, 52 % GC 304 bp mimic (5 pg); lane 36, negative control; lane 37, 900 bp target (5 pg); lane 38, 40 % GC 304 bp mimic (5 pg); lane 39, 44 % GC 304 bp mimic (5 pg); lane 40, 52 % GC 304 bp mimic (5 pg).

3.2.2 The effect of mimic sequence composition on competitive amplification

Although the 52% GC 304 bp mimic could be amplified in isolation it failed to amplify in the presence of the 900 bp target (Figure 3.1, lanes 25-33). Both the 40% GC and 44% GC 304 bp mimics were successfully amplified in the presence of the 900 bp target (45% GC) (lanes 2-11 and 13-23 respectively), except in lanes 2 and 13. Due to the large amount of target present in these reactions, the mimic was out-competed resulting in the loss of signal observed.

3.2.3 An examination of target and mimic amplification efficiencies

Due to lack of amplification observed using the 52% GC 304 bp mimic in competitive reactions and the inability to calculate initial starting ratios using this mimic, it was decided to determine if there were any differences in amplification efficiency between this and the other mimics when amplified in isolation. Figure 3.2 shows the fluorescence profiles generated when selected target and mimics were amplified in isolation. From these profiles, 5-6 cycles falling within the exponential phase of amplification (as determined by regression analysis) were used to determine the efficiency of amplification as shown in Table 3.2 (Section 2.16.4.1). The amplification efficiencies of the 900 and 600 bp targets were lower than those obtained for the shorter 450 bp target and mimics confirming the decrease in amplification efficiency with target length observed in Section 3.2.1. However, the amplification efficiency of the 52% GC 304 bp mimic was much lower than those obtained for the other targets and mimics. Consequently, work was carried out to determine why this mimic amplified poorly in isolation and not at all in competitive reactions.

3.2.4 The effect of PCR enhancers on competitive amplification

The use of enhancing reagents has been reported to improve the amplification efficiency of GC-rich templates (Hill *et al.*, 1991; Olerup, 1994). To investigate the effect of these reagents on this model system, the 52% GC 304 bp mimic, which did not amplify competitively under normal conditions, was amplified with the 450 bp target in the

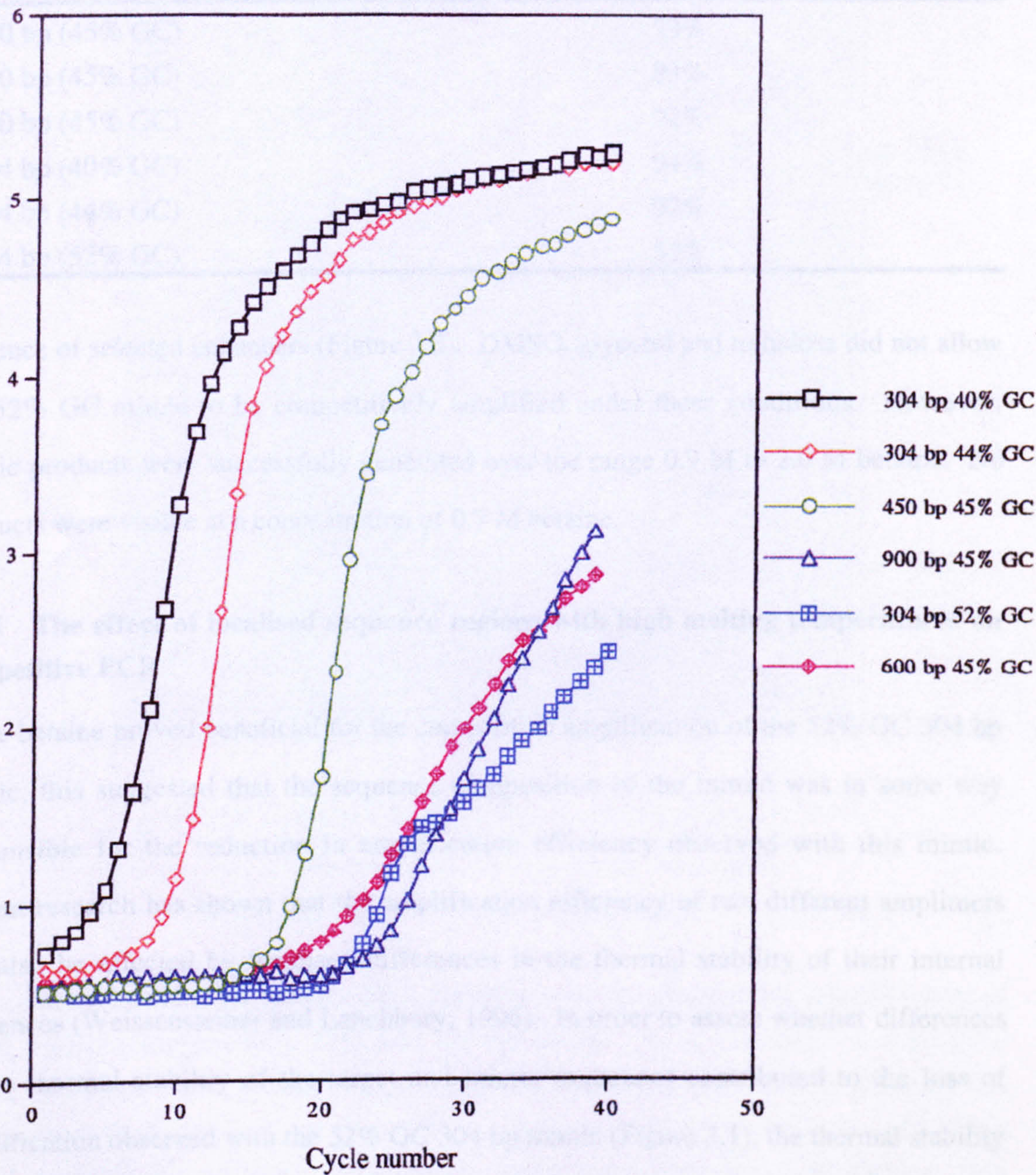


Figure 3.2 Fluorescence profiles generated for the DNA targets and mimics. 5 pg of each target and mimic were amplified for 40 cycles.

Table 3.2 The effect of sequence length on the amplification efficiencies of DNA targets and mimics.

Target / mimic	Amplification efficiency (E)
900 bp (45% GC)	79%
600 bp (45% GC)	83%
450 bp (45% GC)	92%
304 bp (40% GC)	94%
304 bp (44% GC)	92%
304 bp (52% GC)	51%

presence of selected enhancers (Figure 3.3). DMSO, glycerol and trehalose did not allow the 52% GC mimic to be competitively amplified under these conditions. However, mimic products were successfully generated over the range 0.9 M to 2.0 M betaine. No products were visible at a concentration of 0.7 M betaine.

3.2.5 The effect of localised sequence regions with high melting temperatures on competitive PCR

Since betaine proved beneficial for the competitive amplification of the 52% GC 304 bp mimic, this suggested that the sequence composition of the mimic was in some way responsible for the reduction in amplification efficiency observed with this mimic. Recent research has shown that the amplification efficiency of two different amplimers can also be affected by localised differences in the thermal stability of their internal sequences (Weissensteiner and Lanchbury, 1996). In order to assess whether differences in the thermal stability of the target and mimic sequences contributed to the loss of amplification observed with the 52% GC 304 bp mimic (Figure 3.1), the thermal stability profile of the mimic was calculated using a modified version of the programme developed by Poland (Fixman and Freire, 1977). Two regions with localised T_m s in excess of 81°C were observed (Figure 3.4). The first region was located at base positions 143 and 144, while the other larger region occurred between positions 185 and 187. To test this hypothesis further, two additional mimics (304 bp in length) were constructed. One mimic was designed with a higher overall GC content (53%) but lacking

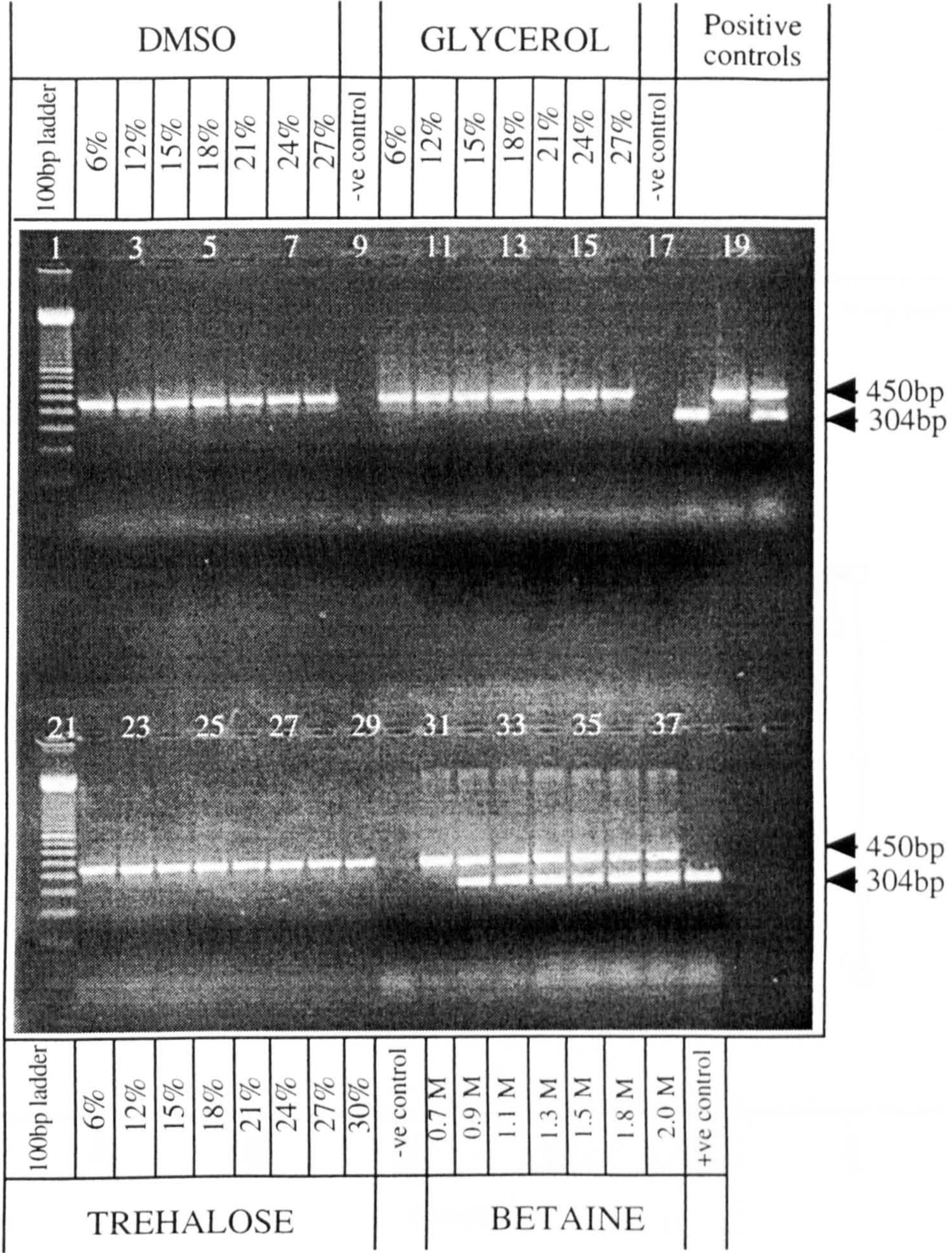


Figure 3.3 The effect of various PCR enhancers on the competitive amplification of a 450 bp DNA target and a 52% GC 304 bp mimic. A constant amount of both the target and mimic (100 pg) were competitively amplified in the presence of selected PCR enhancers. Lane 1, 100 bp ladder; lanes 2-8, samples amplified in the presence of DMSO; lane 9, negative control; lanes 10-16, samples amplified in the presence of glycerol; lane 17, negative control; lane 18, 52% GC 304 bp mimic (100 pg); lane 19, 450 bp target (100 pg); lane 20, 450 bp target (100 pg) and 44% GC 304 bp mimic (100 pg); lane 21, 100 bp ladder; lanes 22-29, samples amplified in the presence of trehalose; lane 30, negative control; lanes 31-37, samples amplified in the presence of betaine; lane 38, 44% GC 304 bp mimic (100 pg). All percentages quoted are final.

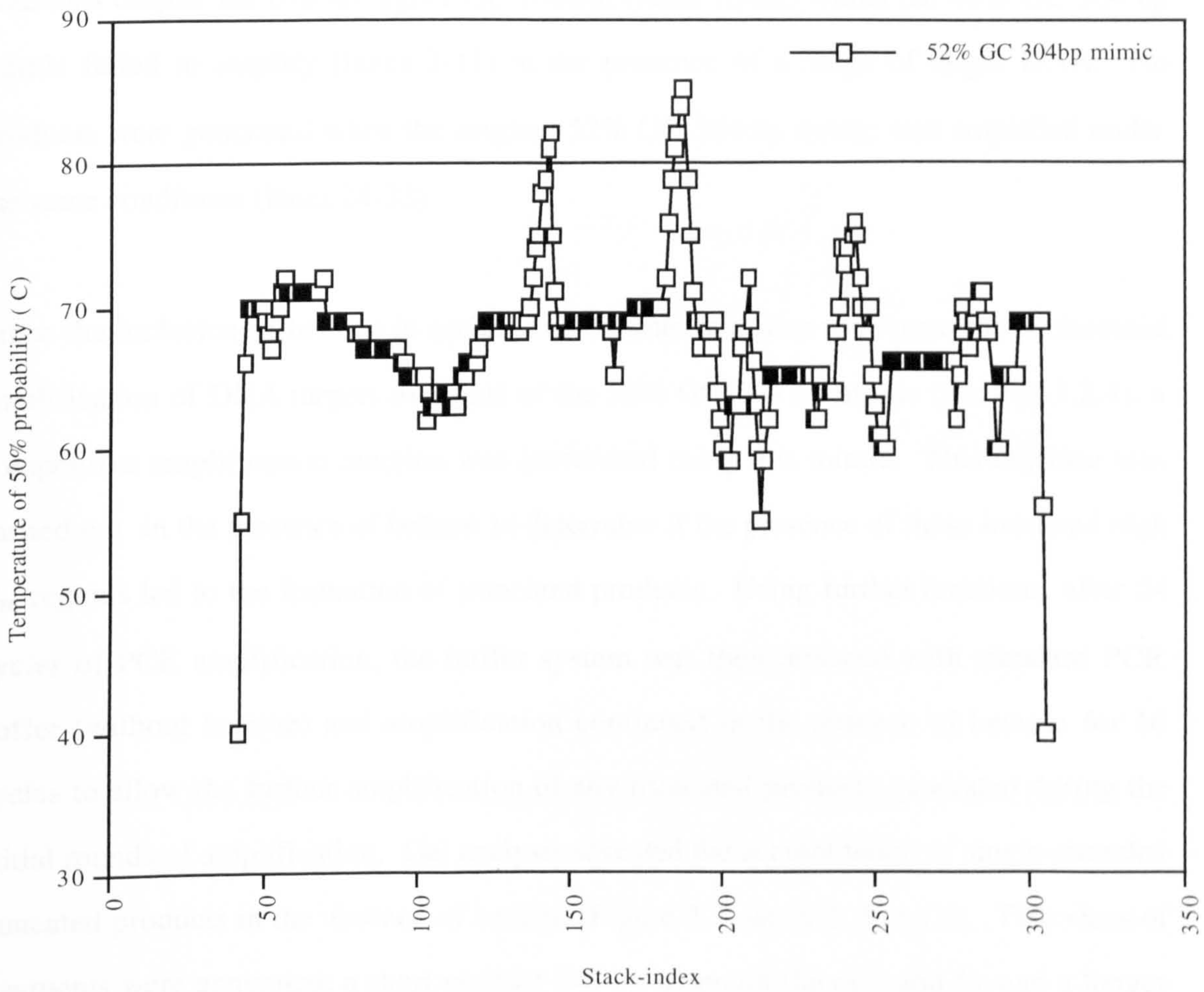


Figure 3.4 Thermal stability profile of the 52% GC 304 bp mimic.

any high T_m domains ($>81^\circ\text{C}$) and a second, with a lower overall GC content (49%) but containing three high T_m domains (Figure 3.5). In Figure 3.6, competitive PCRs using these mimics showed that the 53% GC 304 bp mimic amplified in the competitive reactions despite the overall higher GC content (lanes 13-22) whilst the 49% GC 304 bp mimic failed to amplify (lanes 2-11) in the presence of a range of target DNA. No products were generated when the original 52% GC 304 bp mimic was amplified under the same conditions (lanes 24-33).

Since the inclusion of betaine in earlier experiments was seen to reverse the preferential amplification of DNA targets over that of the 52% GC 304 bp mimic (Section 3.2.4), a competitive amplification reaction was performed using this mimic. This reaction was carried out in the presence of betaine to determine if the presence of these localised high T_m regions led to the formation of truncated products. Using further reactions, after 24 cycles of PCR amplification, the buffer system was then replaced with standard PCR buffer (without betaine) and amplification continued in the absence of betaine for 16 cycles to allow the further amplification of any truncated products generated during the initial rounds of amplification. Gel analysis revealed the accumulation of single-stranded truncated products in the absence of betaine (Figure 3.7, lanes 3, 5 and 6). Two sizes of fragments were generated; a short product 143 bp in length (lanes 3 and 5), and a longer fragment 187 bp in length (lanes 5 and 6).

In order to establish whether or not these high melting temperature regions were indeed responsible for the generation of truncated fragments, these products were isolated, cloned (Section 2.12.3), and their DNA sequences determined (Section 2.17). Examination of the sequence data revealed two features regarding the premature termination events, not only did the termination occur at the high T_m domains, as predicted from the thermal stability profile (Figure 3.4), but this event was also associated with the addition of an extra deoxyadenosine residue at the 3'-end (Figure 3.8).

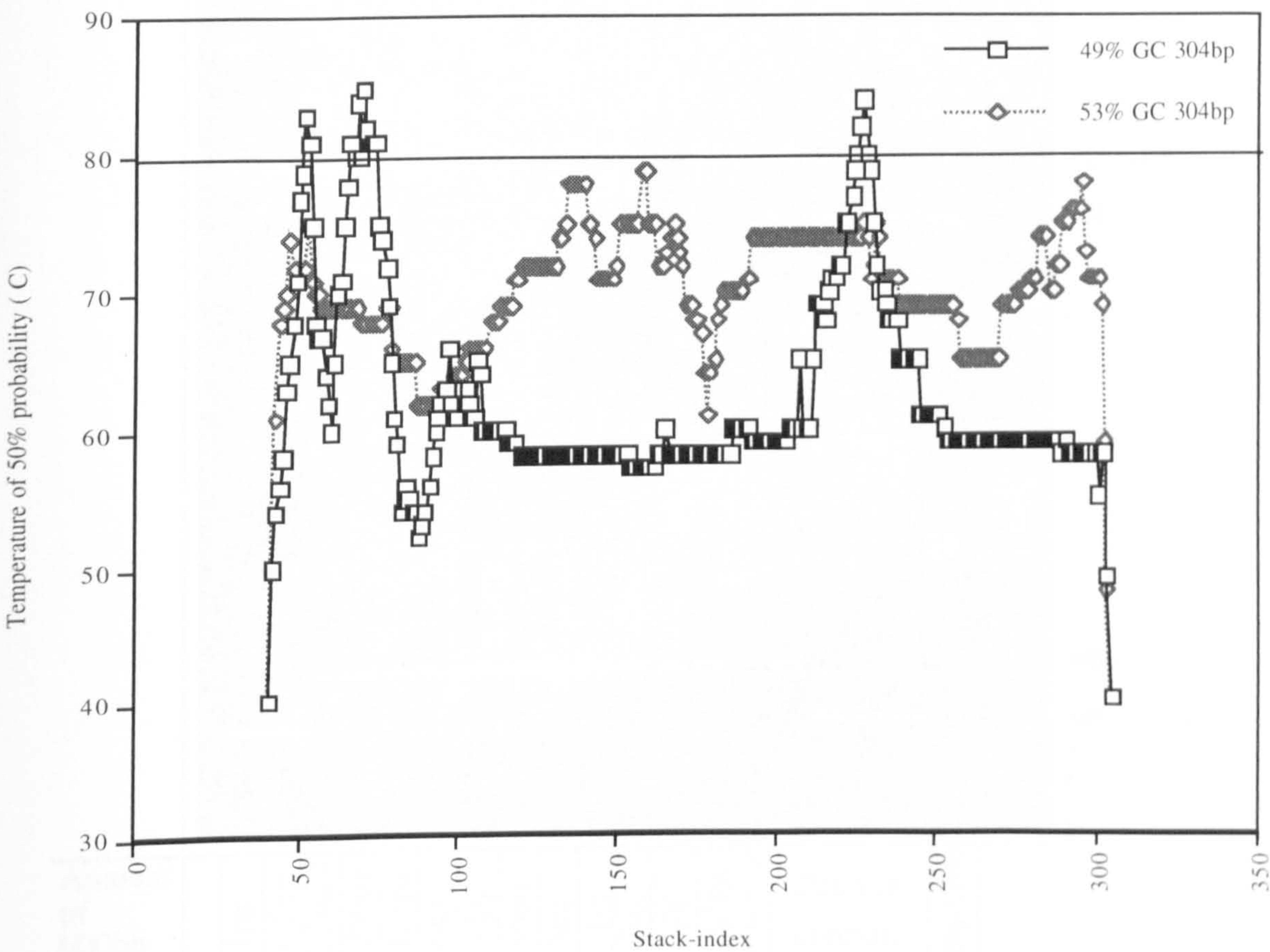


Figure 3.5 Thermal stability profiles of the 49% GC and 53% GC 304 bp mimics.

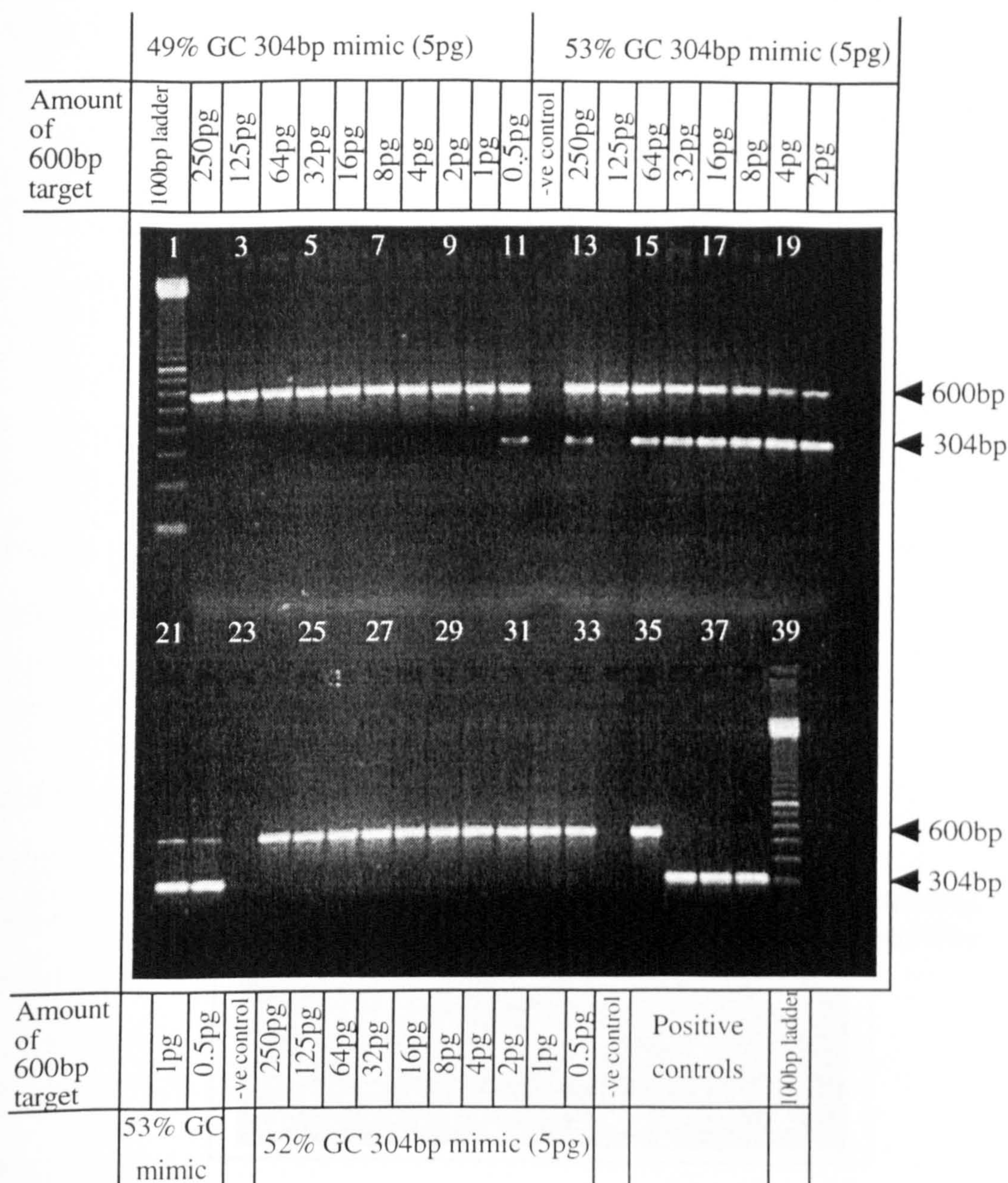


Figure 3.6 Competitive amplification of a 600 bp DNA target in the presence of 304 bp mimics with modified internal sequences. Lane 1, 100 bp ladder; lanes 2-11, dilution series of a 600 bp target amplified in the presence of 5 pg 49% GC 304 bp mimic; lane 12, negative control; lanes 13-22, dilution series of a 600 bp target amplified in the presence of 5 pg 53% GC 304 bp mimic; lane 23, negative control; lanes 24-33, dilution series of a 600 bp target amplified in the presence of 5 pg 52% GC 304 bp mimic; lane 34, negative control; lane 35, 600 bp target (5 pg); lane 36, 49% GC 304 bp mimic (5 pg); lane 37, 53% GC 304 bp mimic (5 pg); lane 38, 52% GC 304 bp mimic (5 pg); lane 39, 100 bp ladder.

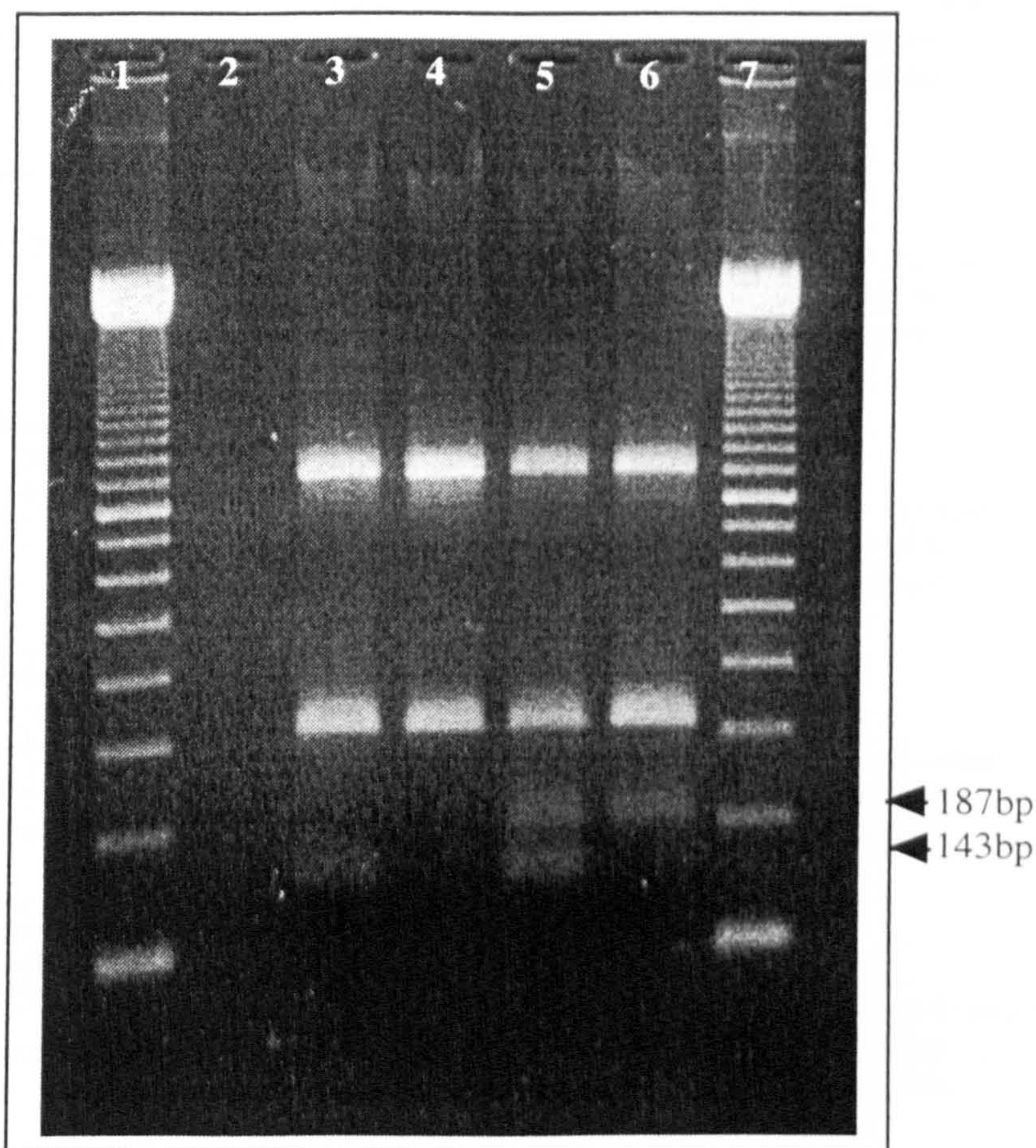


Figure 3.7 Amplification of truncated products following the removal of betaine. Competitive reactions were initially amplified for 24 cycles in the presence of betaine. Following the removal of betaine (lanes 3, 5 and 6), products derived from the first round of amplification were subjected to a further 16 cycles of amplification. Lane 1, 100 bp ladder; lane 2, negative control; lane 4, positive control (900 bp target (100 pg) and 52% GC 304 bp mimic (100 pg)), betaine not removed after initial round of amplification; lanes 3, 5 and 6, 900 bp target (100 pg) and 52% GC 304 bp mimic (100 pg); lane 7, 100 bp ladder.

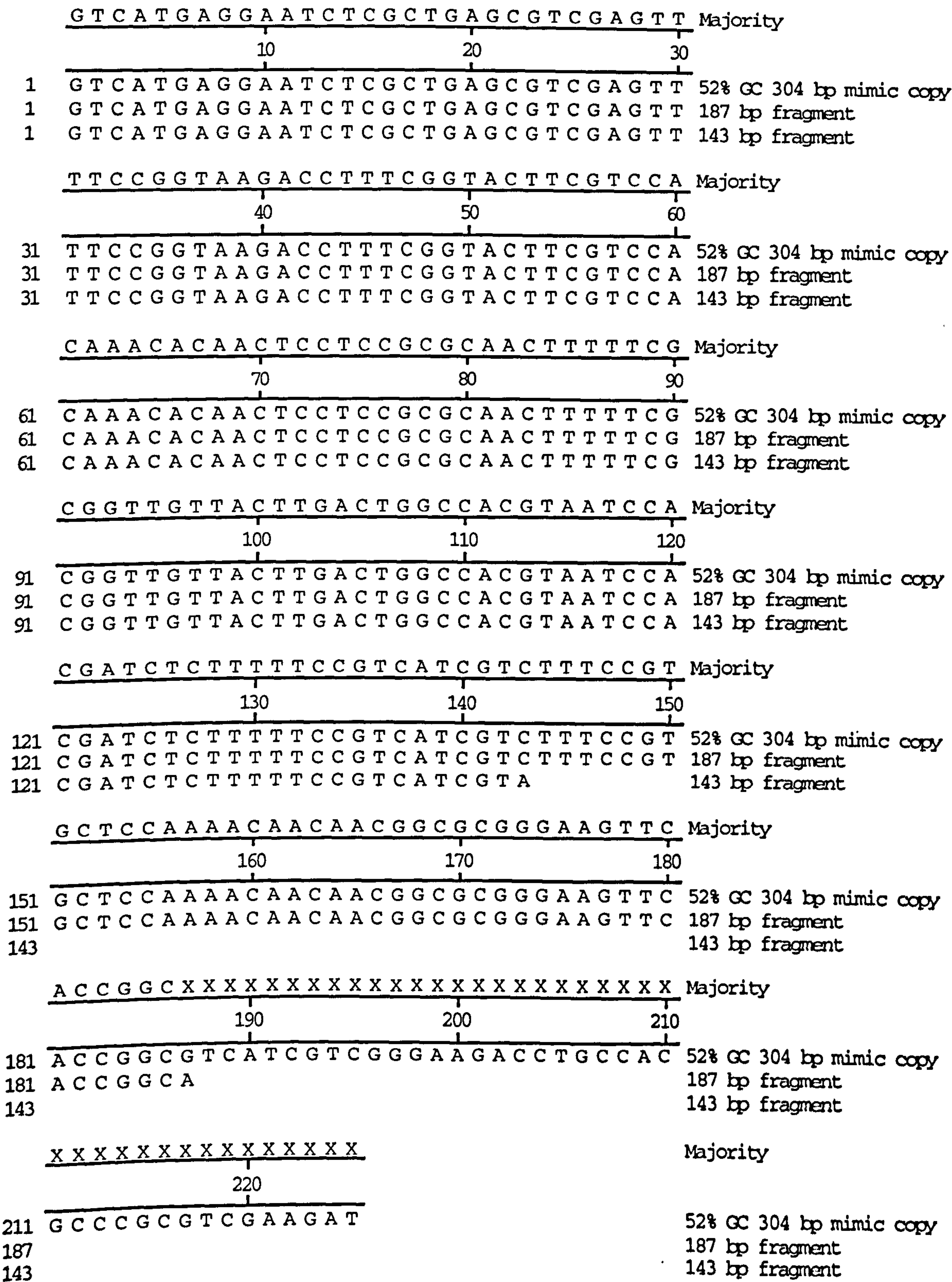


Figure 3.8 Alignment of sequence data showing the termination sites of the truncated PCR products.

This information suggested that either these regions were affecting the processivity of *Taq* DNA polymerase causing the enzyme to “fall off” with the addition of an extra deoxyadenosine residue at the 3’-end or a site-specific misincorporation was responsible for the truncation event.

3.2.6 The effect of high fidelity polymerase mixtures on competitive PCR

To determine if the misincorporation of an extra deoxyadenosine residue was preventing the competitive amplification of the 52% GC 304 bp mimic it was decided to include other heat stable polymerases, possessing 3'-5' exonuclease activity, in the reaction mixtures. This would prevent the potential misincorporation of bases during PCR allowing amplification to continue. Figure 3.9 shows that use of enzyme mixtures (which contained either *Pfu* (Stratagene, Cambridge, UK.) or Vent® (CamBiochem, Cambridge, UK.) DNA polymerases combined with *Taq* DNA polymerase) to the competitive PCR reversed the preferential amplification of a 900 bp target over that of the 52% GC 304 bp mimic (lanes 2-5 and 10-13 respectively). The 52% GC 304 bp mimic failed to amplify competitively when exo⁻ *Pfu* polymerase was used in the enzyme blend (lanes 6-9; Figure 3.9). This particular DNA polymerase has no detectable 3'-5' exonuclease activity to prevent misincorporation during PCR, consequently, the mimic failed to amplify competitively.

3.2.7 The effect of localised sequence regions, with high melting temperatures, on the amplification of long DNA templates.

The inclusion of enzyme mixtures in 'long PCR' has been shown to increase the efficiency of the extension reaction, which had been previously limited by the incorporation of mismatched base-pairs by *Taq* DNA polymerase (Barnes, 1994). To confirm that localised high melting temperature regions were responsible for the site-specific misincorporation (and hence poor amplification) observed in competitive PCR it was decided to investigate whether these regions affected other variants of PCR such as long range amplification.

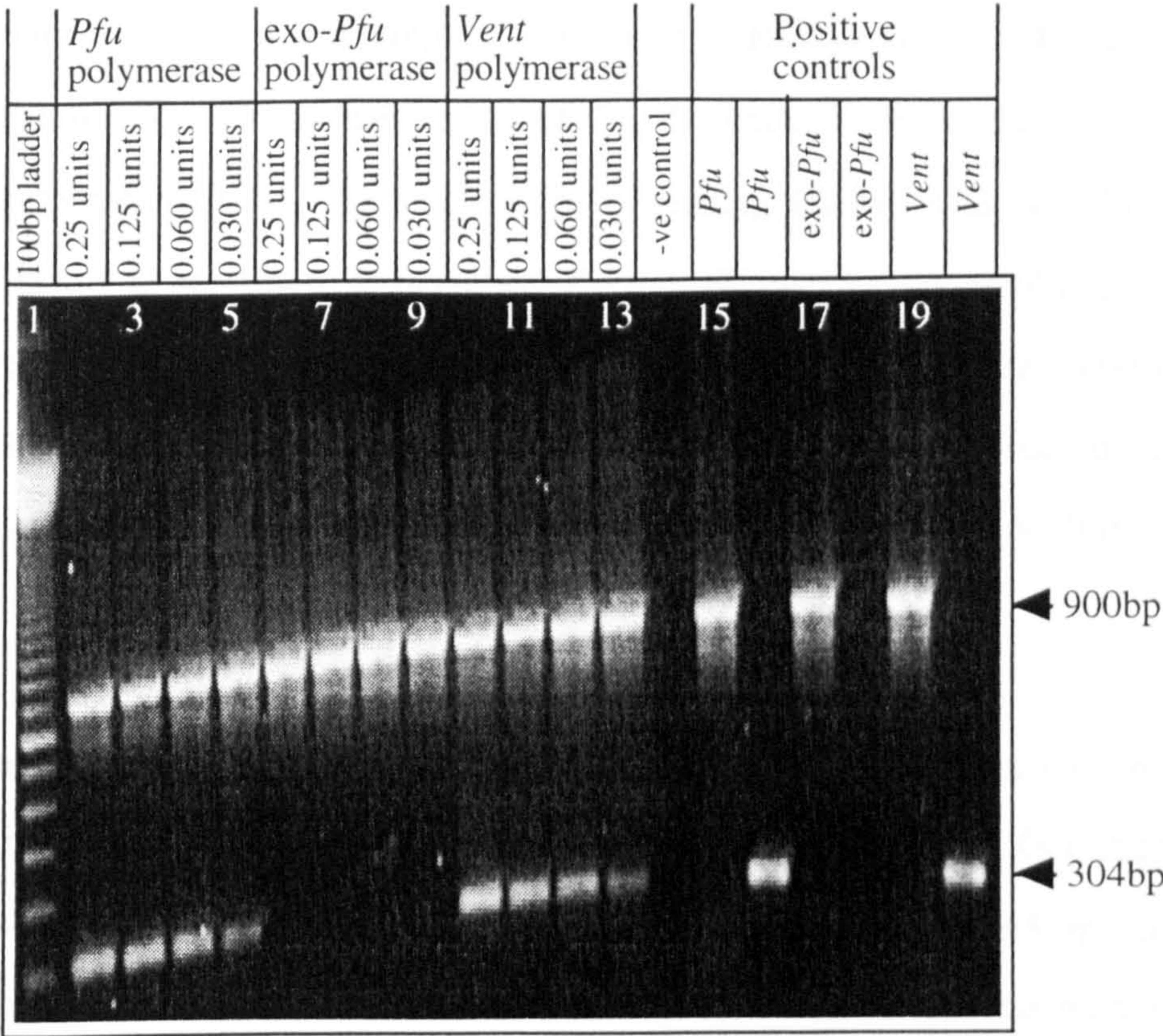


Figure 3.9 The effect of high fidelity DNA polymerases on the competitive amplification of a 900 bp target and a 52% GC 304 bp mimic. 100 pg of a 900 bp target and a 52% GC 304 bp mimic were competitively amplified in the presence of varying concentrations of high fidelity polymerases. Lane 1, 100 bp ladder; lanes 2-5, serial dilution of *Pfu* polymerase in the presence of 0.625 U *Taq* polymerase; lanes 6-9, serial dilution of exo-*Pfu* polymerase in the presence of 0.625 U of *Taq* polymerase; lanes 10-13, serial dilution of Vent[®] polymerase in the presence of 0.625 U of *Taq* polymerase; lane 14, negative control; lane 15, 900 bp target (100 pg) plus 0.25 U *Pfu* polymerase; lane 16, 52% GC 304 bp mimic (100 pg) plus 0.25 U *Pfu* polymerase; lane 17, 900 bp target (100 pg) plus 0.25 units exo-*Pfu* polymerase; lane 18, 52% GC 304 bp mimic (100 pg) plus 0.25 U exo-*Pfu* polymerase; lane 19, 900 bp target (100 pg) plus 0.25 U Vent[®] polymerase; lane 20, 52% GC 304 bp mimic (100 pg) plus 0.25 U Vent[®] polymerase.

To determine if localised GC-rich sequence regions impede the amplification of long DNA templates, it was first necessary to establish conditions under which the generation of long fragments was compromised leading to the generation of truncated products. The λ EMBL 3 vector was chosen as a template to generate PCR fragments of 1 kb and 4 kb in length (Section 2.6). The concentration of *Taq* Extender™ PCR additive (which contains a polymerase with 3'-5' exonuclease proof reading activity) was varied over a range of 0.25 U to 5 U per 100 μ l to determine the cut-off point for the amplification of product. Figure 3.10 shows that at a concentration of 0.5 U *Taq* Extender™ PCR additive per 100 μ l, both 1 kb and 4 kb fragments were generated. Since no PCR products were generated at concentrations below this value, 0.5 U of the additive was used in subsequent investigations.

To demonstrate the effect of localised high melting temperature regions on the amplification of λ DNA, PCR was performed in the presence of betaine. Gel analysis revealed the accumulation of small truncated fragments (approximately 193 bp) in the absence of betaine (Figure 3.11; lanes 12 and 14). Since truncated fragments were only generated during the amplification of the 4 kb fragment, the experiment was repeated to determine if further truncated fragments could be amplified from this particular sequence of the template. An additional truncated fragment (approximately 3.8 kb in length), to that of the 193 bp fragments formed previously, was generated (Figure 3.12; lane 6).

The truncated fragments were isolated, cloned and the sequences determined by cycle sequencing to confirm the site of termination. All the shorter fragments were found to be 193 bp in length and possessed the same sequence. Subsequent analysis of the last 395 bp of the large fragment showed that the termination event occurred at the same base position as that for the much shorter fragment (Figure 3.13). Also in the majority of cases an extra deoxyadenosine residue was added to the 3'-terminus of the truncated fragment showing non-random termination.

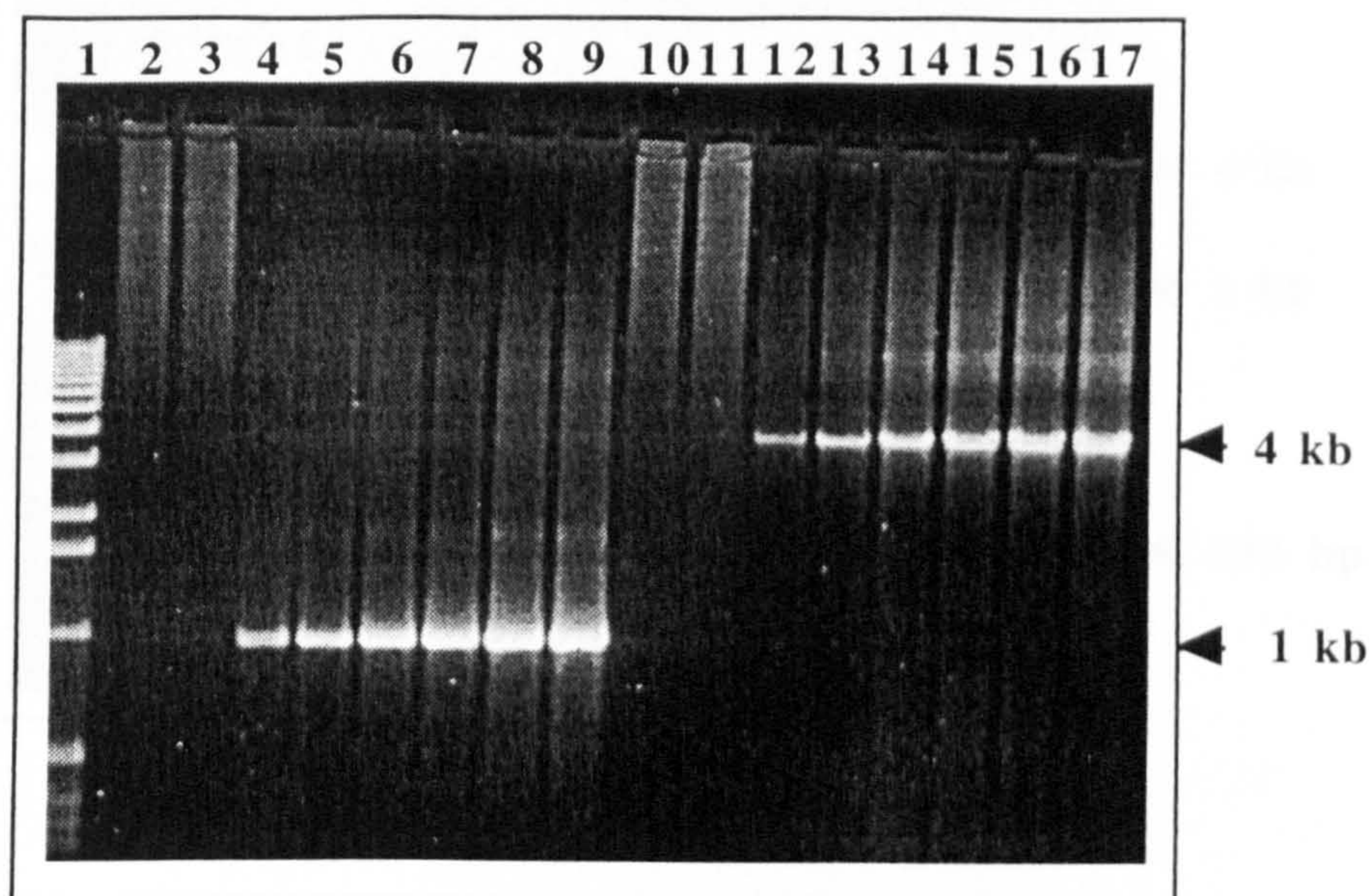


Figure 3.10 The effect of *Taq* Extender™ PCR additive concentration on the amplification of 1 kb and 4 kb λ DNA targets. Lane 1, 1 kb ladder; lane 2, negative control; lanes 3-9: (1 kb λ DNA target amplified in the presence of increasing amounts of *Taq* Extender™ PCR additive); lane 10, negative control; lanes 11-17, (4 kb λ DNA target amplified in the presence of increasing amounts of *Taq* Extender™ PCR additive).

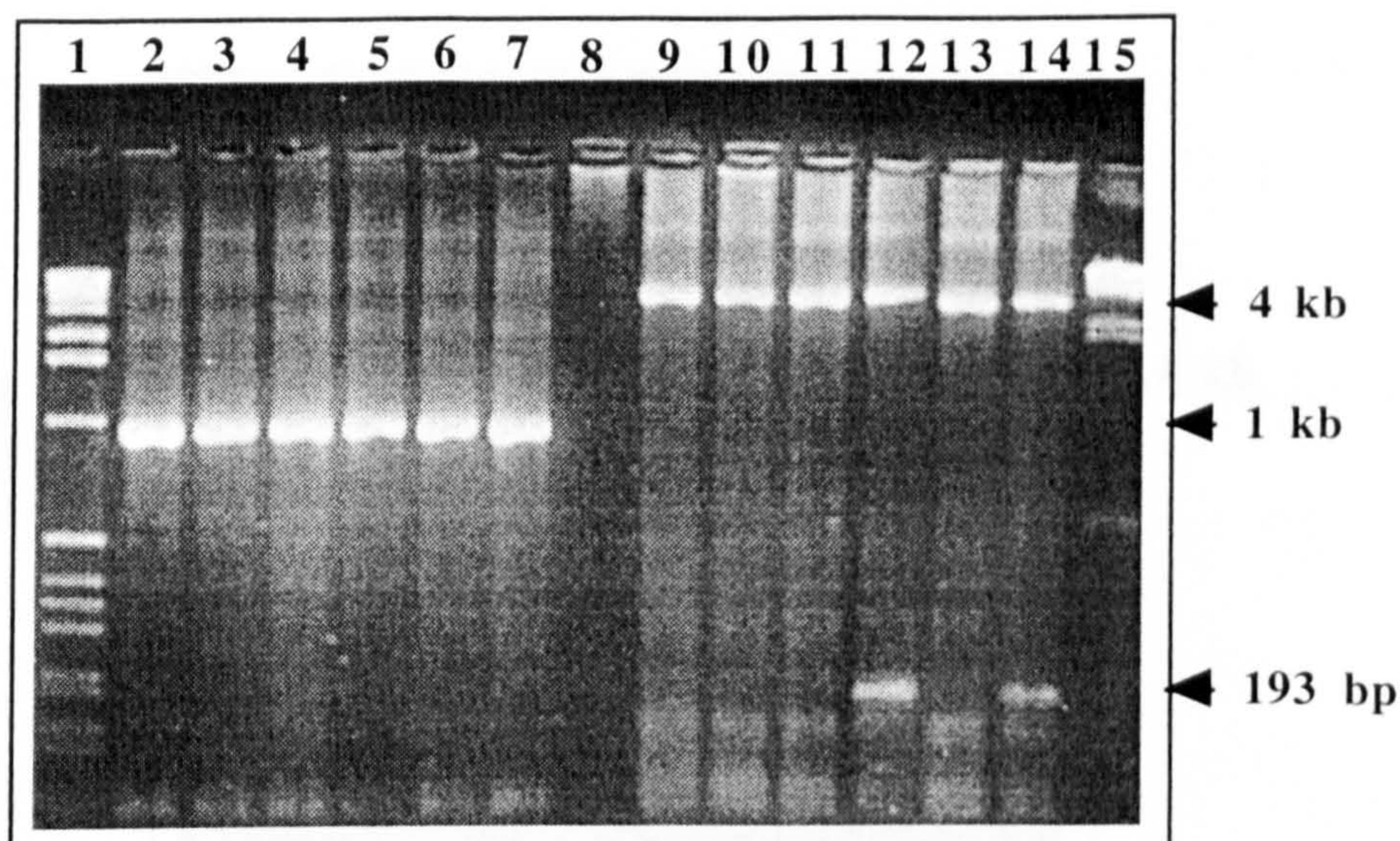


Figure 3.11 Generation of truncated fragments following the amplification of 1 and 4 kb λ DNA targets in the absence of betaine. Targets were amplified initially for 24 cycles in the presence of betaine. Following the removal of betaine, the samples were reamplified for a further 16 cycles. All reactions were carried out in triplicate. Lane 1, 1 kb ladder; lanes 2 and 3: (products after 24 and 40 cycles, respectively); lanes 4 and 5: (products after 24 and 40 cycles, respectively); lanes 6 and 7: (products after 24 and 40 cycles, respectively); lane 8, negative control; lanes 9 and 10: (products after 24 and 40 cycles, respectively); lanes 11 and 12: (products after 24 and 40 cycles, respectively); lanes 13 and 14: (products after 24 and 40 cycles, respectively); lane 15, λ DNA/*Hind* III markers.

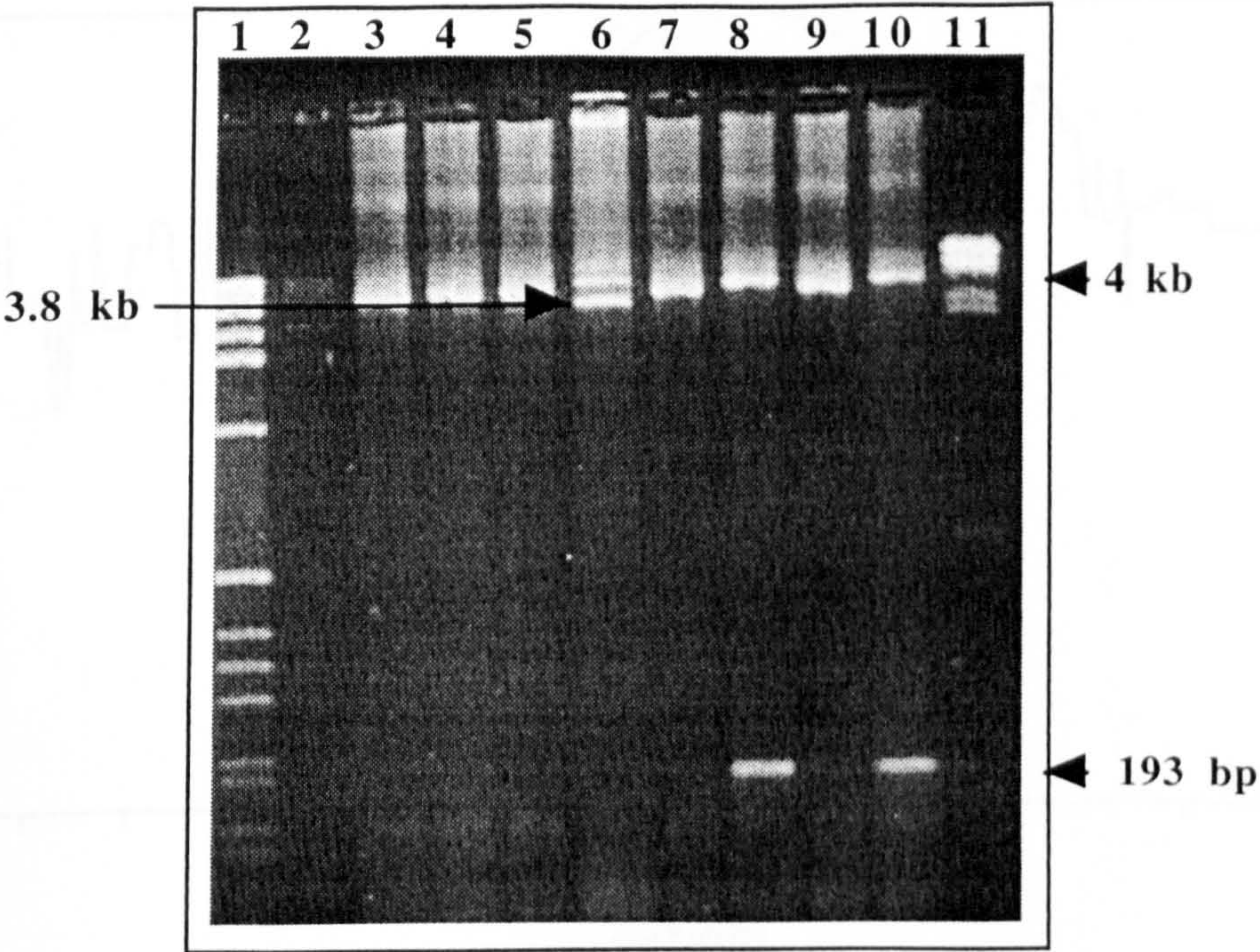


Figure 3.12 Generation of truncated fragments following the amplification of a 4 kb λ DNA target in the absence of betaine. The target was amplified for 24 cycles in the presence of betaine. Following the removal of betaine, samples were reamplified for a further 16 cycles. Lane 1, 1 kb ladder; lanes 2, negative control; lanes 3 and 4: positive control after 24 and 40 cycles, respectively; lanes 5 and 6 (products after 24 and 40 cycles, respectively); lanes 7 and 8: (products after 24 and 40 cycles, respectively); lanes 9 and 10: (products after 24 and 40 cycles, respectively); lane 11, λ DNA/Hind III markers.

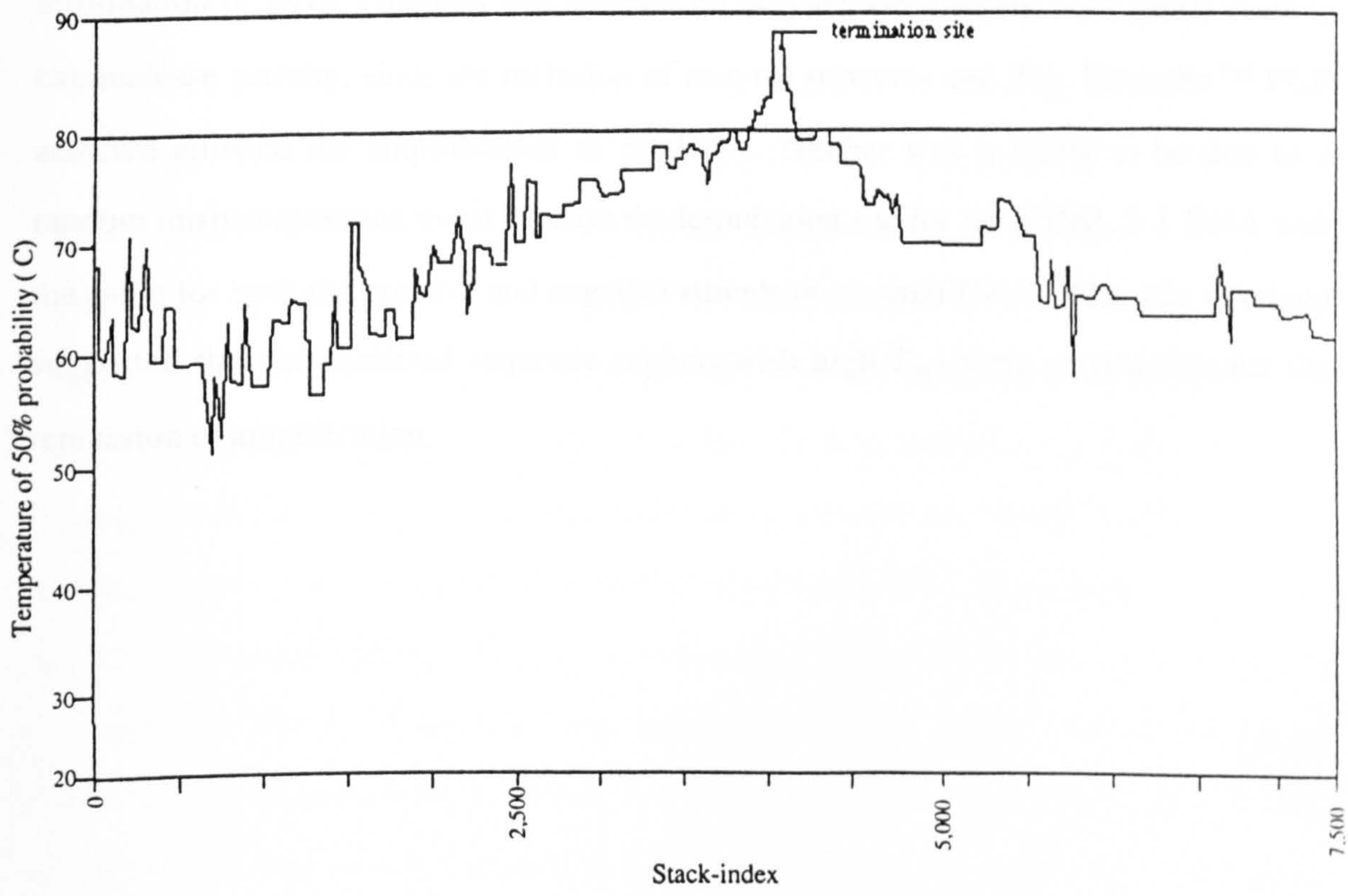


Figure 3.13 Thermal stability profile of the first 15 kilobases of the λ EMBL 3 vector showing the site of termination.

Although sequence length was seen to effect amplification efficiency, it appears that localised sequence regions (with high melting temperatures) constitute a far more serious impediment to the competitive amplification of the 52% GC 304 bp mimic and the generation of long PCR products under sub-optimal conditions in long PCR. Premature termination of DNA synthesis was specific for polymerases lacking 3'-5' proof reading exonuclease activity, since the inclusion of enzyme mixtures and *Taq* Extender™ PCR additive allowed the amplification of products. Neither was it likely to be due to a random misincorporation event because the termination site for the EMBL 3 λ DNA was the same for both the positive and negative strands of plasmid DNA. The data obtained suggested that the localised sequence regions with high T_m s were responsible for the repression of amplification.

3.3 Discussion

An important finding of this investigation was that the amplification of a PCR mimic during competitive PCR (which amplifies correctly in isolation) can be prevented by the presence of other DNA targets. Since both the 52% GC 304 bp mimic and the DNA targets shared the same primer binding sites, the lack of amplification could not have been due to the sequence of the primers. By studying how the required starting ratios varied with the % GC content (constant length) and with length (constant % GC), it was found that increased length or higher GC content led to a reduced amplification efficiency (Table 3.4). Determination of the amplification efficiencies of both the targets and mimics by real-time analysis confirmed the reduction in the efficiency of amplification observed with target length (Table 3.5). However, the results obtained in Section 3.2.3 do not suggest that an inverse exponential relationship between amplification efficiency and template length exists, as described by McCulloch *et al.*, (1995). Consequently, the small differences in amplification efficiency observed using this model system could be readily compensated for by using the appropriate correction factor (Section 2.16.1). Furthermore, the amplification efficiency of the 304 bp mimic with the highest % GC content (52%) was greatly reduced compared to mimics of the same size. The higher overall % GC content may result in either an overall stiffening effect on the helix or an increased chance of localised higher T_m regions with associated stiffness which may slow polymerase extension and prevent competitive amplification of the mimic. The preferential amplification of one target over another, due to differences in sequence composition, may affect all PCR-based assays and is therefore of fundamental importance.

A number of DNA secondary structures have been reported to cause problems in PCR including hairpins and G-tract quadruplexes. Stable template hairpins generally lead to the generation of truncated PCR products (Chou, 1992; Shen and Hohn, 1992). No deletions were observed when the 52% 304 bp mimic was routinely amplified competitively in the presence of various DNA targets suggesting that hairpins were not

responsible for the decrease in the amplification efficiency of this mimic. G-rich motifs have been shown to inhibit primer extension by *Taq* DNA polymerase by forming quadruplex structures during cycle sequencing (Woodford *et al.*, 1995). Similarly, CGG repeats can block DNA synthesis *in vitro* (Henke *et al.*, 1997; Usdin and Woodford, 1995). Analysis of the template sequence at the termination site using the programme, Oligo, (Version 4, National Biosciences, Inc., Plymouth, USA.) did not reveal the presence of any G-tract quadruplexes, CGG-rich motifs or stable hairpin structures which could account for the reduction in amplification efficiency observed with the mimic.

Although the potential effects of these structures cannot be underestimated, from the results obtained (Sections 3.2.2 and 3.2.3), it was evident that some other feature of the mimic template was having a significant affect on the ability of the 52% GC 304 bp mimic to amplify competitively in the presence of varying sized DNA targets. Of the co-solutes tested, only betaine had any visible effect on the amplification of the 52% GC 304 bp mimic. Betaine binds to and stabilises AT base-pairs whilst destabilising GC base pairings resulting in a net specific destabilisation of GC-rich regions (Weissensteiner and Lanchbury, 1995). The use of PCR enhancers (Baskaran *et al.*, 1996; Hill *et al.*, 1991; Olerup, 1994) has also been found to be beneficial when a template possessed localised sequence regions whose T_m exceeded 81°C. The thermal stability of a target template can be expressed as a function in which the equilibrium temperature at which half the base pairs at each position are denatured is plotted against their stack index (position from the 5' end of the fragment) (Steege, 1994). When the thermal stability profile of the 52% GC 304 bp mimic (Figure 3.4) was calculated using a modified version of the programme developed by Poland (Fixman and Freire, 1977), two regions with localised T_m s in excess of 81°C were observed. The first region was located between base position 143 and 144 of the stack index while the other, larger region occurred between positions 185 and 187. The presence of these localised sequence regions with T_m values in excess of 81°C suggests that these regions may be responsible for the suppression of amplification. During the denaturation step of the PCR, the template strands would usually denature at

the melting domains leaving localised sequence regions, possessing high T_m values, denaturing only partially or not at all. This heterogeneous denaturation would inhibit the ability of *Taq* DNA polymerase to read through these regions leading to the generation of truncated amplification products.

This inability of *Taq* DNA polymerase to read through regions of high localised T_m was thought to be the result of the combined effect of a lack of a 3'-5' exonuclease activity and its tendency to terminate polymerisation by the addition of a deoxyadenosine residue. To investigate this hypothesis further, other DNA polymerases were examined which possess 3'-5' exonuclease proof reading activity. The inclusion of enzyme mixtures in "long PCR" has been shown to increase the efficiency of the extension reaction, which had been previously limited by the incorporation of mismatched base-pairs by *Taq* DNA polymerase (Barnes, 1994). Whilst this is more likely to be significant with longer targets, it was decided to examine if a similar effect could be demonstrated with the 52% GC mimic.

This hypothesis was supported by the ability of the enzyme mixtures (containing *Taq* and thermostable polymerases with 3'-5' exonuclease activity) to amplify the mimic in competitive PCR by preventing the misincorporation of bases during PCR (Section 3.2.6). Misincorporation events, such as the addition of the deoxyadenosine residue, caused by the presence of localised sequence regions with high melting temperatures, can be overcome thus allowing amplification to continue.

The data obtained from the amplification of long DNA templates (Section 3.2.7) confirmed the significance of base misincorporation in preventing the efficient amplification of long DNA targets and suggests that it was a specific event dependent upon the target sequence rather than a random feature dependent on the natural fidelity of the polymerase. Premature termination of DNA synthesis was specific for proof reading polymerases possessing 3'-5' exonuclease activity, since the inclusion of the *Taq*

Extender™ PCR additive (which possesses 3'-5' exonuclease activity) allowed the amplification of products. However, premature termination was unlikely to be due to a random misincorporation event because the termination site was the same for both the normal and complementary strands of DNA. These results demonstrate that the termination site, which comprises a localised sequence region with a T_m of 88°C, constituted a serious impediment to the generation of long PCR fragments under sub-optimal conditions.

Most sequence extension studies on DNA polymerases have focused on the process of elongation. However, despite the fact that polymerase activity needs to be rapid and accurate for effective function, the problem of pause sites has yet to be satisfactorily addressed (Mytelka and Chamberlain, 1996). *In vitro* replication studies of ϕ X174 DNA with T4 DNA polymerase demonstrated the formation of truncated products resulting from stable hairpin structures in the sequence (Challberg and Englund, 1979) but truncation was not observed with *E. coli* DNA polymerase I. The term "pause-site" has recently been defined as a temporary stop in processivity resulting from localised sequence. This implies, given sufficient time, that the processivity of the polymerase would be restored and the enzyme would then continue to produce full length products. However, an erroneous incorporation of an extra base caused by the local sequence at the pause site, would be highly resistant to further elongation as with amplified refractory mutation style assays (Newton, 1989). The ϕ X174 pause sites were postulated to result from secondary structures in the sequence. Similar results were found with four forms of the *E. coli* DNA polymerase III each of which showed differences in pause sites (LaDuca *et al.*, 1983). This could potentially result from differences in the ability of the various forms of the enzyme to read through particular regions or their potential for erroneous incorporation at these sites.

Whilst a number of the secondary structures mentioned earlier have been reported to cause pausing of DNA elongation, other pause sites which lack secondary structures have

been observed. As these sites cannot be associated with spatially separated sequences (secondary structures) it was postulated that specific primary sequences could also cause this effect (Weaver and De Pamphilis, 1982). Some of the primary sequences responsible for extension arrest are made up of stretches of alternating purine and pyrimidine residues (Weaver and DePamphilis, 1984). Such sequences have been shown to adopt a left hand helical conformation (Wells *et al.*, 1982), forcing the polymerase to pause until equilibrium results in the formation of a right-handed helix. Ten such residues have been shown to be strong arrest sites (Weaver and DePamphilis, 1984).

In the system described here, the sequence of the 52% GC 304 bp mimic does not suggest this type of pause site which results from the localised formation of a left-handed helix. Regions of high T_m and GC rich regions may exert their effect by producing localised, rigid regions of DNA sequence resistant to unwinding which inhibit the polymerase. The reversal of polymerase pausing brought about by betaine observed in this study may be caused by two potential mechanisms. A weak non-co-operative binding of betaine with AT-pairs in the major groove of the B-form of the double helix serves to stabilise this form (Rees *et al.*, 1993) and move equilibrium in this direction, whilst increasing the hydration of the molecule leads to an overall improvement in flexibility (Mytelka and Chamerberlain, 1996). This is thought to facilitate a conformational change in the enzyme-DNA-dNTP complex which is a limiting step in polymerisation and serves as an additional check for correct base pairing since only correct base pairs can induce a rapid isomerisation of the ternary complex leading to catalysis of the nucleotide phosphoryl-transfer reaction (Johnson, 1993). Betaine also exerts an isostabilising effect on the differential thermal stability of A:T and G:C base pairs. This isostabilising effect results from the large destabilising of betaine on G:C pairs whilst a lesser destabilising effect is exerted on A:T pairs (Rees *et al.*, 1993), presumably resulting from the binding of betaine to these residues.

In conclusion there appears to be a variety of DNA sequences and structures which may render regions of DNA inaccessible to a DNA polymerase. The subsequent pausing may result in termination of delayed extension depending on the system used. The significance of the pausing will also vary according to the system.

CHAPTER 4

THE APPLICATION OF CAPILLARY ELECTROPHORESIS AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ACCURATE QUANTIFICATION OF COMPETITIVE PCR PRODUCTS

4. THE APPLICATION OF CAPILLARY ELECTROPHORESIS AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ACCURATE QUANTIFICATION OF COMPETITIVE PCR PRODUCTS

4.1 Introduction

As competitive PCR gains wider application, there is a concomitant requirement to develop methods which not only provide accurate quantitative analysis of competitive PCR products but also limit the labour-intensive analysis of products generated by multiple titration assays. A large variety of procedures are available for the detection and quantification of amplification products (reviewed in Section 1.2.2). PCR products of different sizes are usually separated by gel electrophoresis (Menzo *et al.*, 1992; Piatak *et al.*, 1993; Zimmermann *et al.*, 1994). For quantification of the respective DNA bands, a video analysis system can be used (Sundfors and Collan, 1995). Radioactively or fluorescently-labelled deoxynucleotide triphosphates (Dostal *et al.*, 1994; Furtado *et al.*, 1993) or oligonucleotides (Arnold *et al.*, 1992; Wang *et al.*, 1989) have also been used for the quantification of PCR products. However, there is some debate over the accuracy of results obtained using such assays (Zimmermann and Mannhalter, 1996).

High performance liquid chromatography (HPLC) (de Kant *et al.*, 1994) and capillary electrophoresis (CE) (Arawawa *et al.*, 1993; Butler *et al.*, 1995; Landers *et al.*, 1993) have been used for the analysis of PCR amplified products and numerous other molecules of biological origin (Landers *et al.*, 1993). To date there have been limited reports of these techniques being applied to the quantification of PCR products (de Kant *et al.*, 1994; Kolesar *et al.*, 1995; Lu *et al.*, 1994; Rossomando *et al.*, 1994). Of the several reports describing the application of HPLC to the analysis of PCR products, non-porous

resin-based, anion-exchange column packings were adapted for analysis but required re-equilibration intervals between runs (Chang *et al.*, 1994; de Kant *et al.*, 1994; Katz and Haff, 1990). More recently, ion-pair, reverse-phase HPLC (IR-RP-HPLC) on alkylated non-porous poly(styrene)divinylbenzene particles has been shown to be well suited to rapid quantification of dsDNA fragments by on-line UV absorbance (Huber *et al.*, 1993) or fluorescence detection (Oefner *et al.*, 1994). The earlier uses of CE for quantification purposes used laser-induced fluorescence for detection purposes and relied on the comparison of the peak area from the sample with those obtained for a range of simultaneously amplified standards.

Although it is important to minimise any size differences between the target and mimic (discussed in Chapter 3), using a similar sized mimic to that of the *Legionella* target template would involve employing a mimic 900 bp in length. This in itself could pose problems (McCulloch *et al.*, 1995), consequently it was decided to use a smaller 304 bp mimic in the competitive PCR assay. Furthermore, to minimise the effect of sequence length and composition on the amplification efficiencies of both the target and mimic, a mimic 284 bp in length and a 304 bp target were employed in reactions to examine the effect of target length on the accuracy of the assay.

The aim of this study was therefore to determine the suitability and level of accuracy that could be achieved using a competitive PCR system by employing either IP-RP-HPLC or CE to quantify different sized targets by measuring the point at which the amount of mimic added produces equal molar amounts of both target and mimic products (the equivalence point).

4.2 Results

4.2.1 Quantification of competitive PCR products using CE

Figure 4.1 (Panel A) shows the peak areas of the *Hae* III /pGEM DNA standard digest (Hewlett-Packard, Watford, Herts., UK.) used to generate the calibration curve for the quantification of the competitive PCR products. Four replicate injections of the standards were performed to determine the apparent mobility of the analyte. This was determined using Equation 5:

$$\mu_a = \frac{I \times L}{t \times V}$$

Equation 5: Where μ_a = apparent mobility (cm^2/Vs); L = total capillary length (cm); I = effective capillary length (inlet to window, cm); t = migration time (sec) and V = applied voltage (volt).

The electrophoretic pattern obtained when a ten-fold dilution series of the 900 bp target was competitively amplified in the presence of a constant amount of a 304 bp mimic (10 pg) is shown in Figure 4.1 (Panels B to F). The same samples, analysed by agarose gel electrophoresis, are shown in Figure 4.2. The equivalence point of the target and mimic can be readily observed as the relative ratios of the products generated during PCR progress from mainly target to mainly mimic products.

Equation 6 was used to determine the volume of *Hae* III/pGEM standard digest injected onto the capillary:

$$\text{volume} = \frac{\pi \cdot d^2 [(\mu_a) \cdot (V) \cdot (\text{Int})]}{L}$$

Equation 6: Where: d = diameter of capillary (cm); μ_a = apparent mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$); V = voltage used for injection (V); Int = injection time (seconds) and L = total length

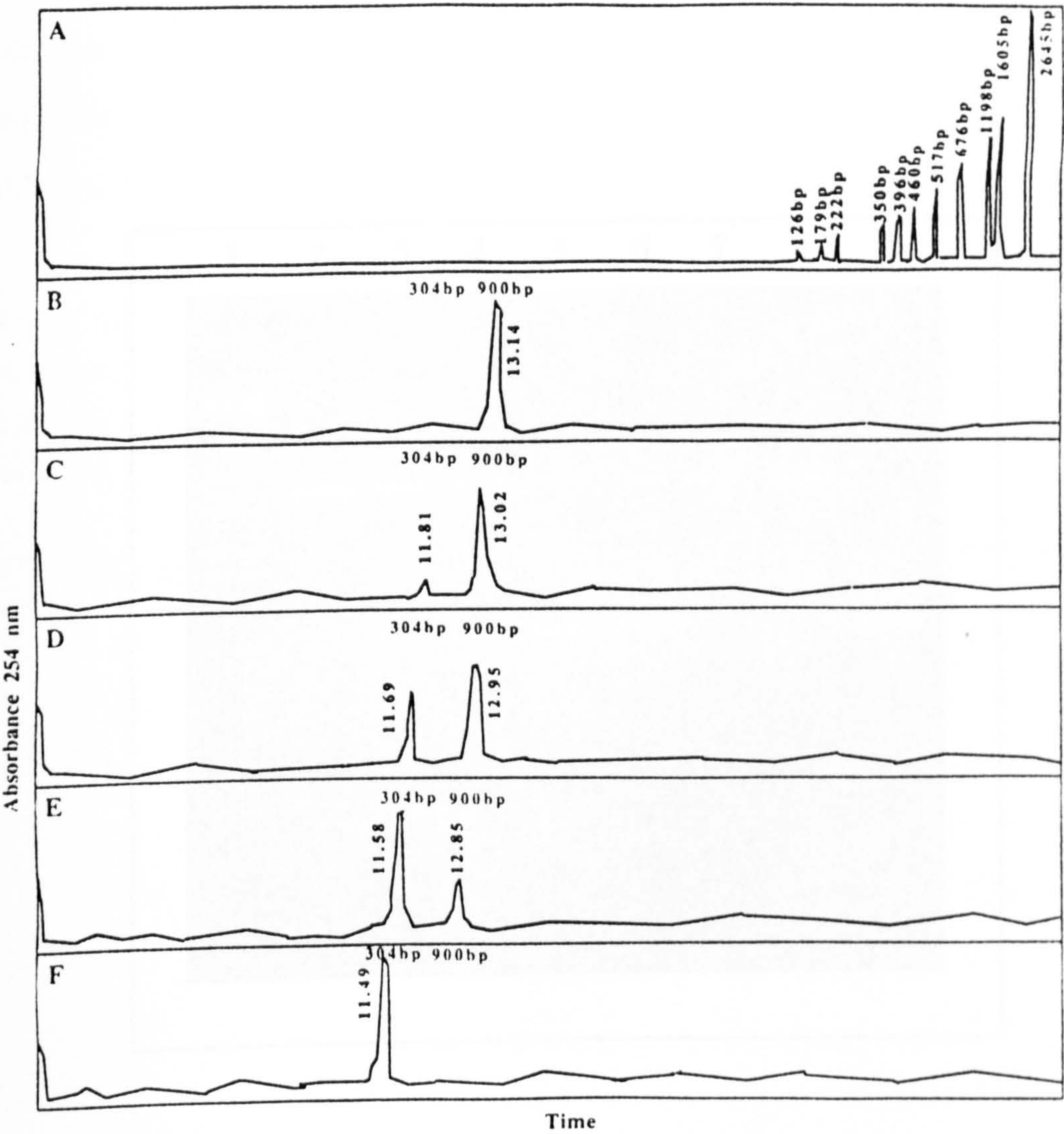


Figure 4.1 CE analysis of competitive PCR products. Panel A: *Hae* III/pGEM DNA standards used for the generation of the calibration curve; Panels B-F, Electropherograms of PCR products; the amounts of target (900 bp) given were amplified in the presence of a constant amount of mimic (300 bp): 1 ng target (Panel B); 100 pg target (Panel C); 10 pg target (Panel D); 1 pg target (Panel E); 100 fg target (Panel F). The absorbance profiles were obtained using a wavelength of 254 nm.

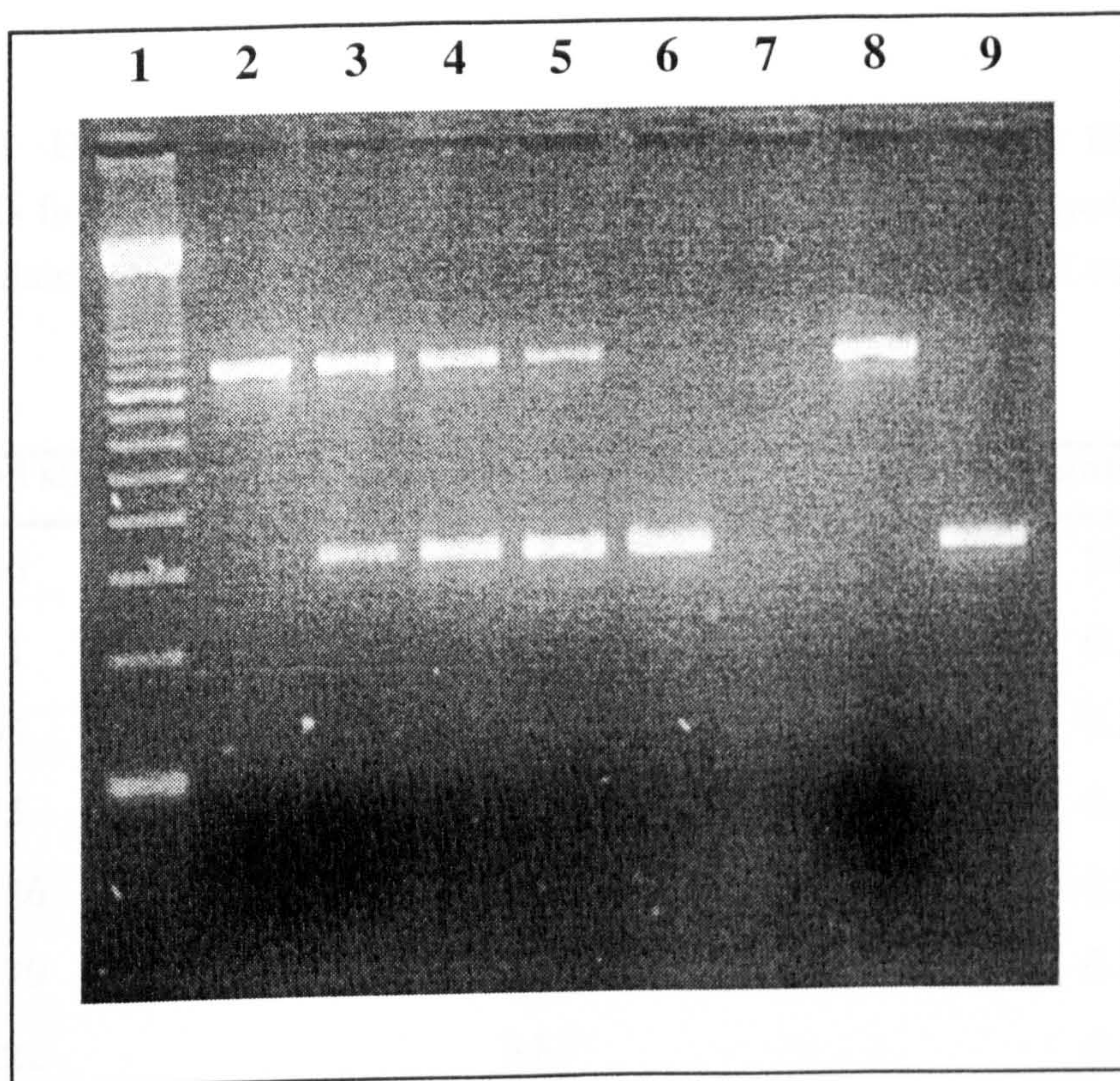


Figure 4.2 Agarose gel analysis of competitive PCR products. Lane 1, 100 bp marker ladder; lane 2, 1 ng target; lane 3, 100 pg target; lane 4, 10 pg target; lane 5, 1 pg target; lane 6, 100 fg target; lane 7, negative control; lane 8, 900 bp target control (10 pg); lane 9, 304 bp mimic control (10 pg). The target was competitively amplified in the presence of 10 pg mimic.

of the capillary (cm).

From the volume of the standards injected, it was possible to calculate the amount of standard loaded on to the capillary. Dividing the total amount of pGEM DNA loaded onto the column by the peak areas generated by each of the *Hae* III/pGEM standards allowed the amount of DNA present in each standard to be determined as in Table 4.1.

Table 4.1 Determination of the amount of DNA present in *Hae* III/pGEM DNA standards following CE analysis. The amount of DNA present in each standard peak was calculated to generate a calibration graph for the quantification of competitive PCR products.

Band size (bp)	Area of peak	Amount of DNA (ng)
36	0.688	0.087
51	0.830	0.10
65	1.23	0.16
75	1.30	0.17
126	2.08	0.26
179	3.06	0.39
222	3.83	0.48
350	6.12	0.77
396	6.89	0.87
460	8.04	1.01
517	8.88	1.12
676	11.88	1.50
1198	19.89	2.51
1605	31.90	4.20
2645	47.24	5.96

Figure 4.3 shows the standard peak areas plotted as a function of the amount of DNA present in each standard. Since the relationship between peak area and the amount of DNA present in each standard was linear ($r^2 = 1.000$), then comparison of the peak areas of the unknown sample with those of standards of known concentration could be used to determine the amount of DNA present in a competitive PCR. Table 4.2 shows the initial amounts of 900 bp DNA target and 304 bp mimic present in each of the samples as well as the amounts of each PCR product generated following amplification. This table demonstrates that PCR products of differing sizes could be readily quantified allowing the initial amount of target present in the sample to be calculated from the ratio of the final amount of target and mimic products.

Table 4.2 Quantification of competitive PCR products using CE. The amount of each PCR product was calculated using the calibration graph derived by the capillary electrophoresis of *Hae* III/pGEM DNA standards of known concentration.

Initial amount of target and mimic added	Area of peak	Amount of PCR product present (ng)
1. 900 (1 ng)	8.85	1.12
304 (10 pg)	2.52	0.32
2. 900 (100 pg)	6.12	0.77
304 (10 pg)	2.73	0.34
3. 900 (10 pg)	5.55	0.70
304 (10 pg)	6.73	0.85
4. 900 (1 pg)	2.46	0.31
304 (10 pg)	8.12	1.02
5. 900 (100 fg)	1.23	0.15
304 (10 pg)	10.79	1.36

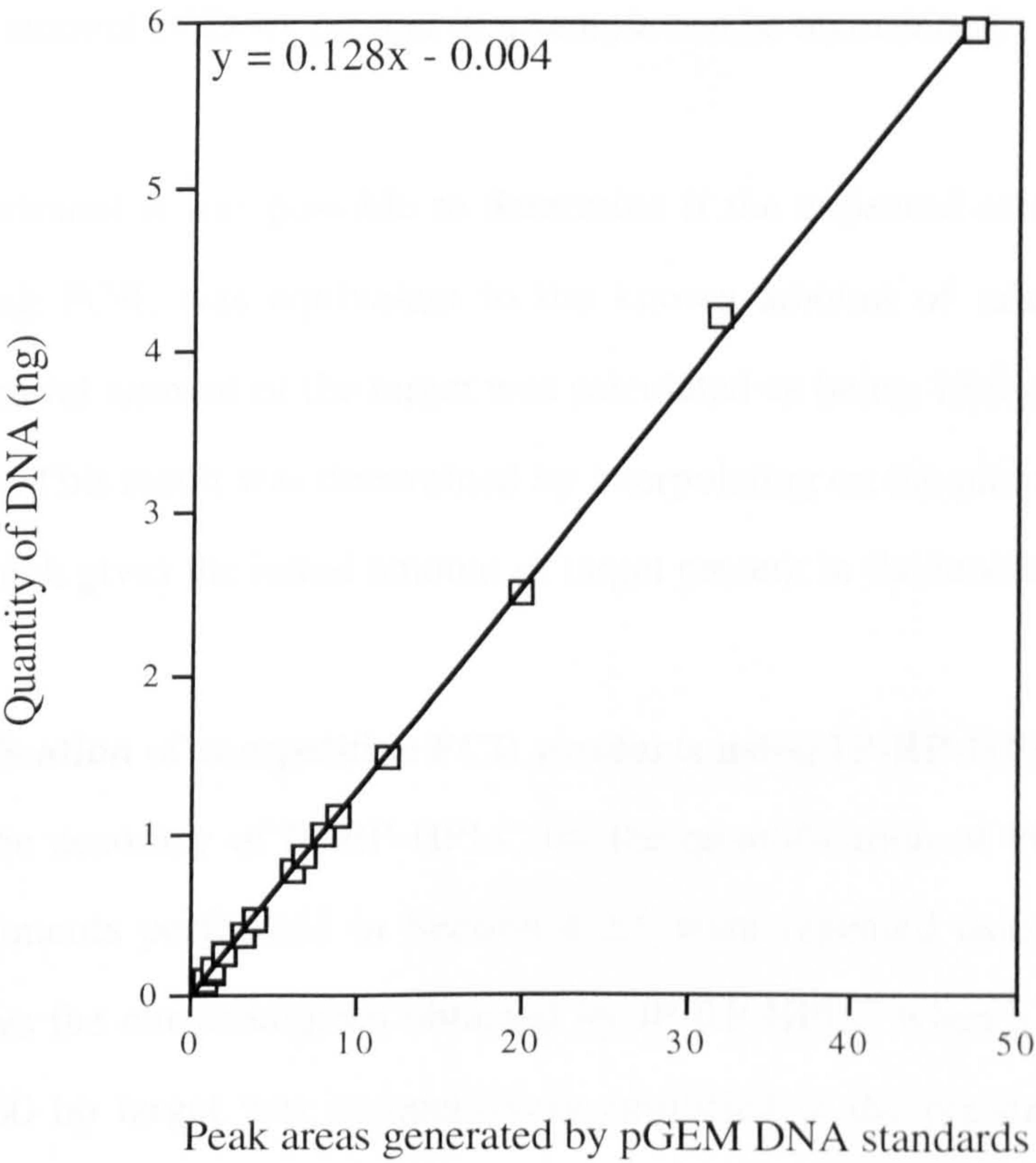


Figure 4.3 Standard graph showing the relationship between the peak area and the amount of DNA present in the *Hae* III/pGEM DNA standards following CE analysis.

For CE analysis, a known dilution series of the 900 bp target was prepared and amplified in the presence of a constant amount of mimic (10 pg). The equivalence point of the target and mimic was determined by plotting the log of the ratios of amplified target to mimic products as a function of the log of the initial amount of target added to the PCR as described by Piatak *et al.*, (1993) (Figure 4.4). Since the equivalence point of the target and mimic amplification products is assumed to be equal to the initial amounts of target and mimic, the amount of DNA present in a sample can be ascertained.

From this experiment it was possible to determine if the expected concentration of the target, following PCR, was equivalent to the known amount of mimic added to the reaction. The initial amount of the target was calculated as being 15.6 pg in the presence of 10 pg mimic. This result was determined by interpolating on the plot (Figure 4.4) for a Y value of 0 which gives the initial amount of target present in the reaction.

4.2.2 Quantification of competitive PCR products using IP-RP-HPLC

To determine the accuracy of IP-RP-HPLC for the quantification of PCR products, the series of experiments performed in Section 4.2.1 were repeated using this technique. Figure 4.5 shows the chromatogram obtained by IP-RP-HPLC when a ten-fold dilution series of the 900 bp target was competitively amplified in the presence of a constant amount of a 304 bp mimic (10 pg) (Panels B to F). The calibration graph used for the quantification of competitive PCR products was obtained from the peak areas of the *Hae* III/pGEM DNA standards shown in Panel A of Figure 4.5. Figure 4.6 shows the pGEM standard peak areas plotted as a function of amount of DNA.

The amount of DNA present in each standard was determined by integrating the peak areas generated by each of the *Hae* III/pGEM standards and comparing the values

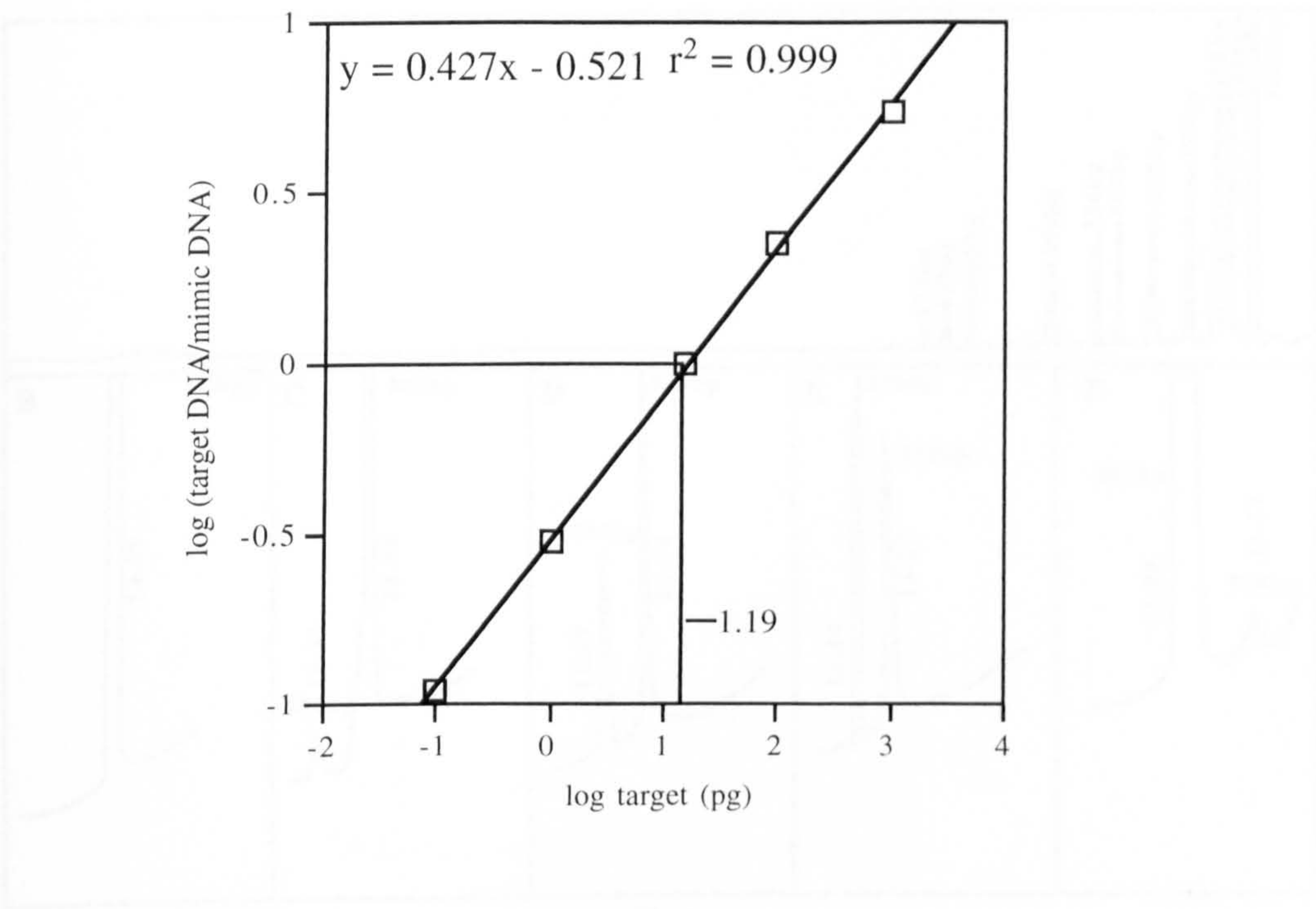


Figure 4.4 Determination of target concentration by competitive PCR and CE.

Figure 4.4 10-RT-HPLC analysis of competitive PCR products. Panel A shows HPLC DNA standards used for the generation of the calibration graph. Panel B shows absorbance profiles of PCR products with 1 ng, 100 pg, 10 pg, 1 ng, 100 pg standards. All target dilutions were subsequently amplified in the presence of 10 pg mimic. The absorbance profiles were obtained using a wavelength of 254 nm.

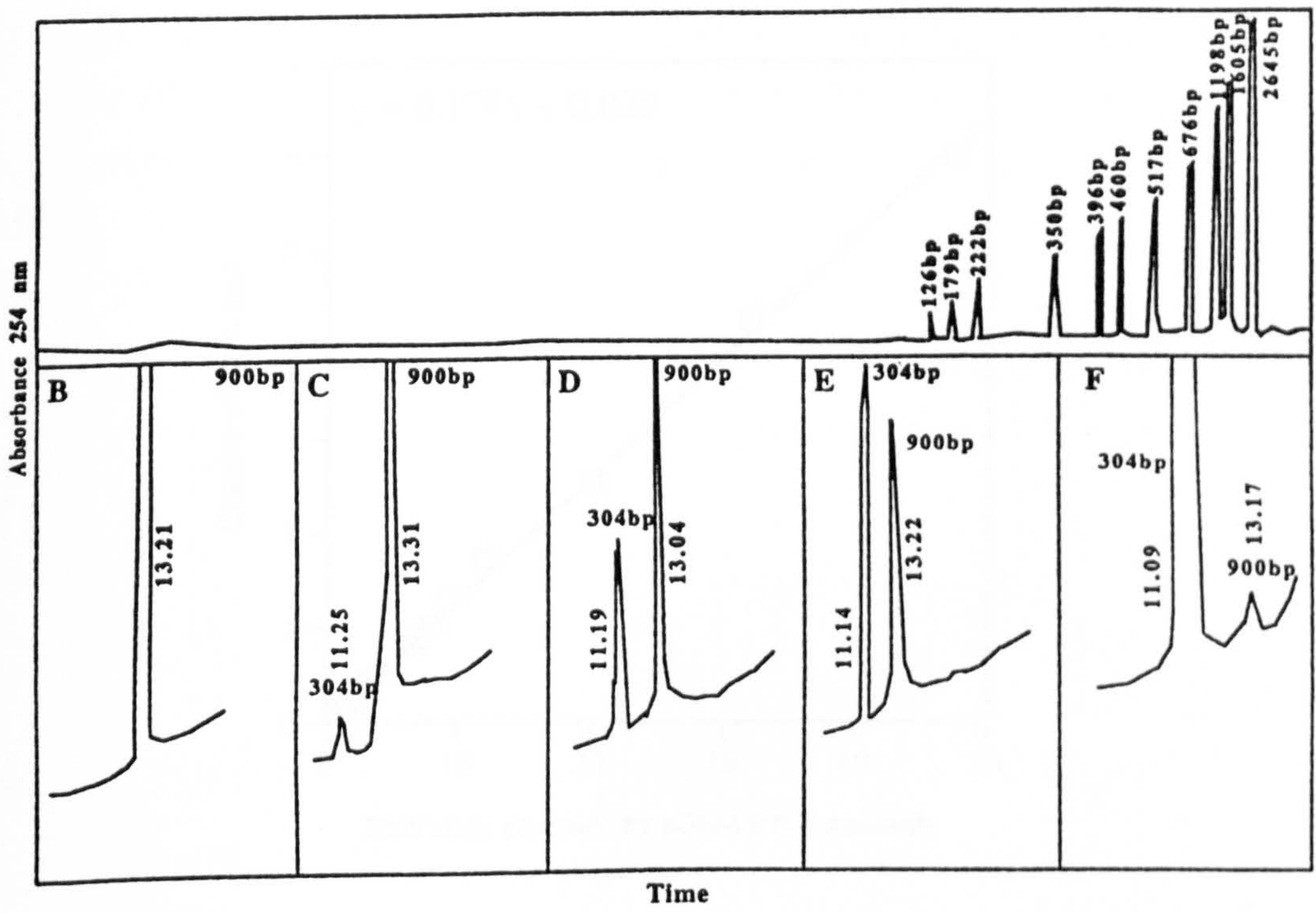


Figure 4.5 Calibration graph showing the relationship between peak area and amount of DNA. The PCR products were competitively amplified in the presence of 10 pg mimic.

Figure 4.5 IP-RP-HPLC analysis of competitive PCR products. Panel A, *Hae* III/pGEM DNA standards used for the generation of the calibration graph; Panels B-F, absorbance profiles of PCR products with:- 1 ng, 100 pg, 10 pg, 1 pg, 100 fg respectively. All target dilutions were competitively amplified in the presence of 10 pg mimic. The absorbance profiles were obtained using a wavelength of 254 nm.

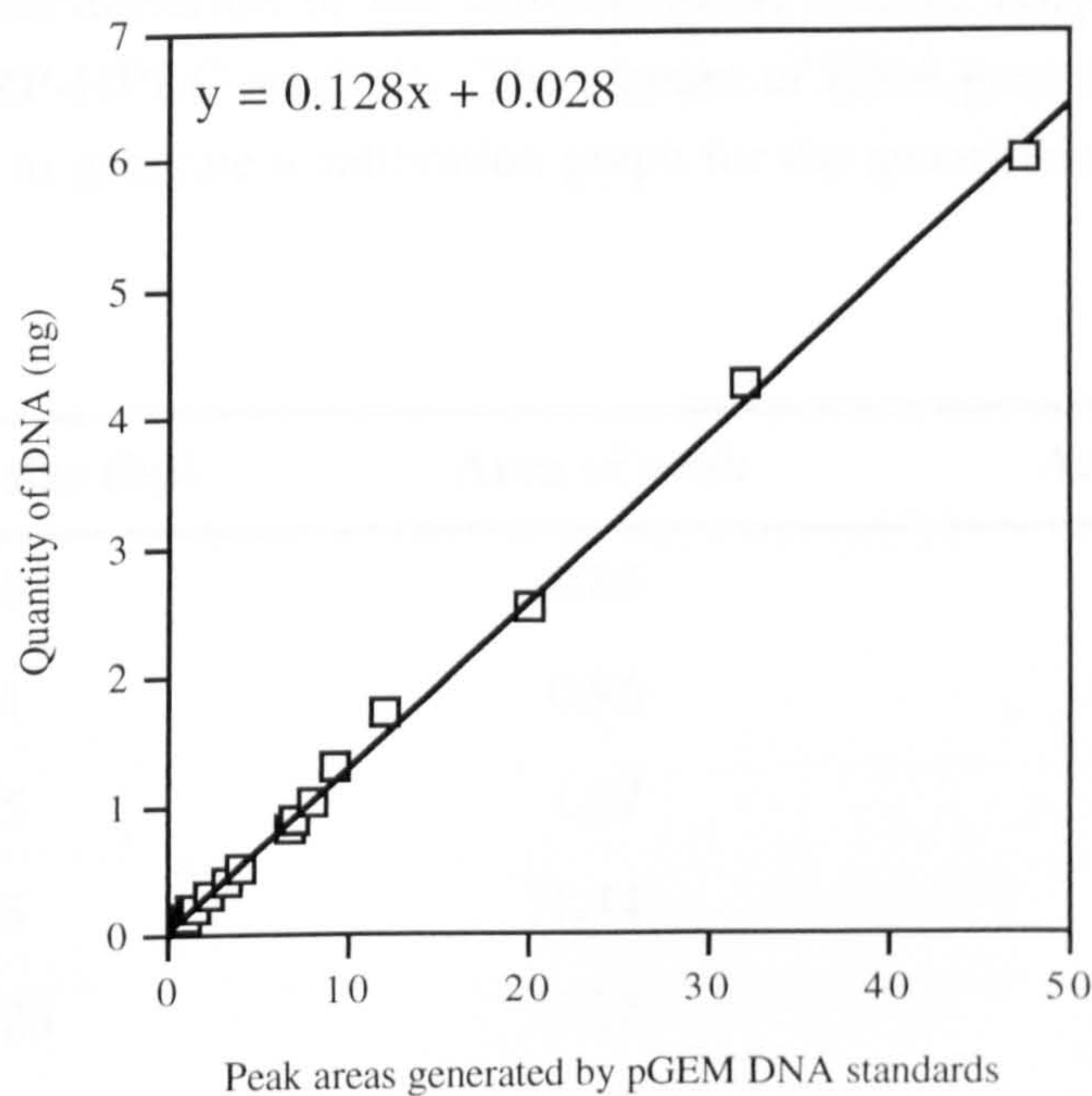


Figure 4.6 Calibration graph showing the relationship between peak area and amount of DNA of the *Hae* III/pGEM standards following IP-RP-HPLC analysis.

obtained with the total amount of standards injected onto the column (Table 4.3). The amount of DNA present in each sample was then calculated using the calibration graph generated from these standards. The initial amounts of both the 900 bp target and 304 bp mimic present in each of the samples is summarised in Table 4.4.

Table 4.3 Determination of the concentration of *Hae* III/pGEM DNA standards following IP-RP-HPLC analysis. The amount of DNA present in each standard peak was calculated to generate a calibration graph for the quantification of competitive PCR products.

Band size (bp)	Area of peak	Amount of DNA(ng)
36	0.83	0.12
51	0.96	0.14
65	1.37	0.19
75	1.44	0.21
126	2.22	0.29
179	3.23	0.42
222	3.96	0.52
350	6.80	0.81
396	7.11	0.91
460	8.09	1.05
517	9.13	1.55
676	12.08	2.50
1198	20.07	2.54
1605	32.05	4.24
2645	47.39	6.02

Table 4.4 Quantification of competitive PCR products using IP-RP-HPLC. The amount of each PCR product was calculated using the calibration graph derived from the IP-RP-HPLC analysis of *Hae* III/pGEM DNA standards.

Initial amount of target and mimic added	Area of peak	Amount of PCR product present (ng)
1. 900 (1 ng)	8.83	1.15
304 (10 pg)	2.54	0.29
2. 900 (100 pg)	6.16	0.81
304 (10 pg)	2.71	0.31
3. 900 (10 pg)	5.61	0.73
304 (10 pg)	6.69	0.82
4. 900 (1 pg)	2.38	0.29
304 (10 pg)	7.99	1.11
5. 900 (100 fg)	1.18	0.12
304 (10 pg)	10.82	1.41

The equivalence point of the target and mimic was determined as in Section 4.2.1 (Figure 4.7), the absolute values obtained for the initial amounts of both the target and mimic being 16.1 pg and 10 pg respectively. The disparity in results between the initial amounts of both the target and mimic was only slightly greater than that obtained using CE.

4.2.3 CE and IP-RP-HPLC analysis of competitive PCR products generated using a target and mimic of similar size

A 284 bp DNA mimic was constructed, which differed in length from the 304 bp target by 20 bp, to minimise the effect of sequence length and composition on the amplification efficiencies of both the target and mimic. Experiments, similar to those employed in the previous sections (4.2.1 and 4.2.2), were conducted using CE and IP-RP-HPLC to determine the accuracy of quantification using a mimic and target of similar sequence

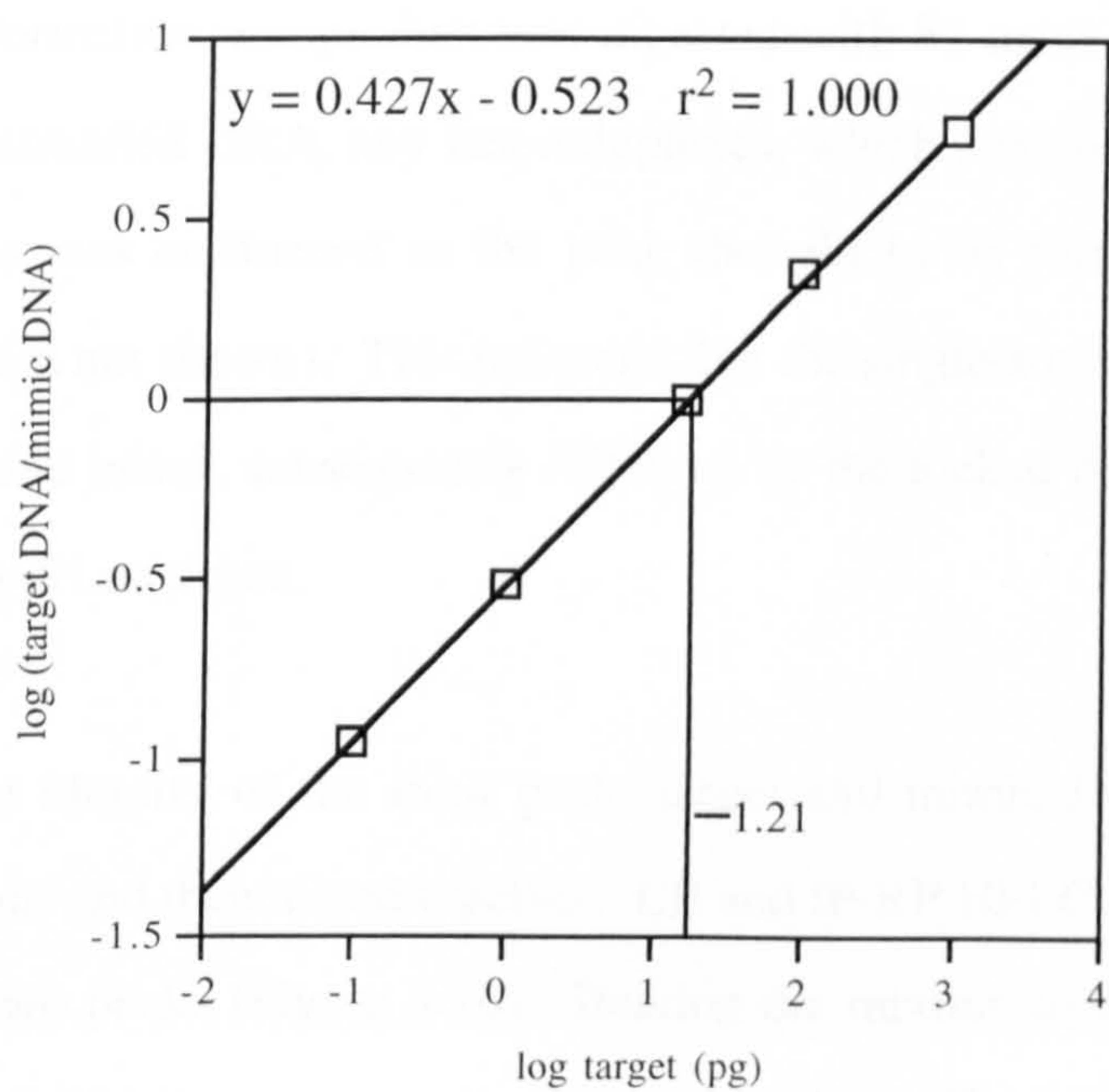


Figure 4.7 Determination of target concentration by IP-RP-HPLC analysis of competitive PCR products. The amount of target present was determined by plotting the log of the ratios of amplified target to mimic products as a function of the log of the initial amount of target added to the PCR.

length and % GC content. The competitive PCR products, resolved by agarose gel electrophoresis, are shown in Figure 4.8. Figure 4.9 shows the electropherograms and chromatograms obtained from the PCR products previously shown in Figure 4.8. In some reactions an additional product peak was observed using CE and IP-RP-HPLC. This was thought to be due to the formation of heteroduplexes because of the similarity between the target and mimic sequences. To determine whether the product was a result of heteroduplex formation, the product was digested with S1 nuclease. Since S1 nuclease digests single-stranded DNA, any heteroduplexes, which contain mismatches, would be digested. This was confirmed as the peak thought to be formed by heteroduplexes disappeared (data not shown). This indicated that the single-stranded loop formed by the unmatched mimic insert, subsequently followed by the nicked double-stranded product, was digested by S1 nuclease.

To confirm the identity of the third peak, target and mimic DNA were amplified in separate reactions and then mixed together. CE and IP-RP-HPLC analysis of this mixture revealed only two peaks (Figure 4.10). Heating the mixture to 94°C for 5 minutes and cooling to 4°C led to the appearance of a new product. The retention time of the new product corresponded to that of the unaccounted peak observed in the original electropherograms and chromatograms obtained by CE and IP-RP-HPLC. There was a concomitant decrease in the peak areas of the target and mimic products.

To determine the effect of heteroduplex formation on quantification, the 304 bp target was co-amplified in the presence of a fixed amount of the 284 bp mimic (10 pg). Plots of the log ratio of 304 bp target and 284 bp mimic products against log of the initial amount of target present generated straight lines with slopes of 1 using both CE and IP-RP-HPLC (Figure 4.11). Ignoring the amount of heteroduplex product formed during the reaction (as would be the case with gel electrophoresis where no heteroduplex products are visible, Figure 4.8) a mean initial target concentration of 18.6 pg was obtained using CE

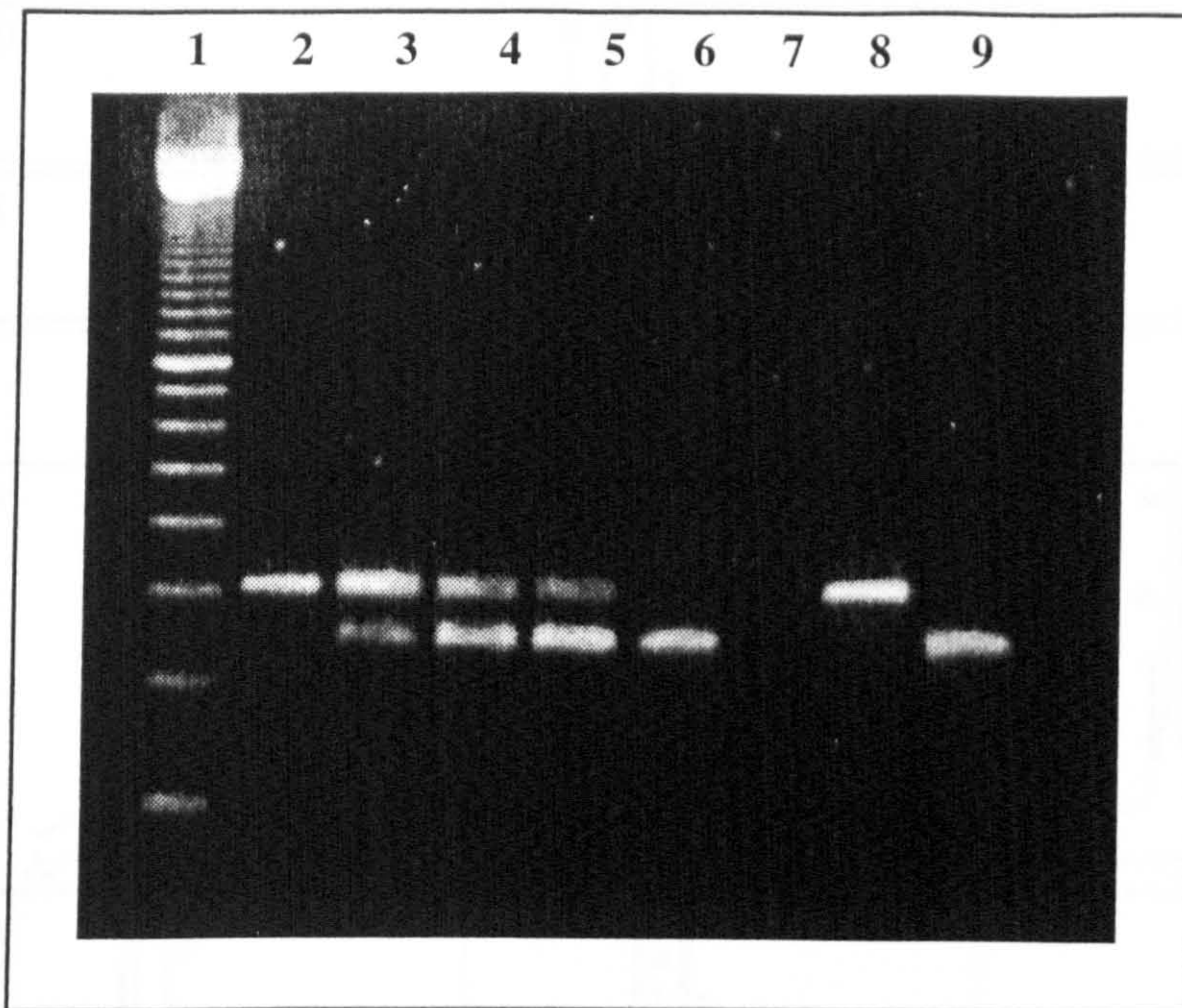


Figure 4.8 Agarose gel analysis of competitive PCR products using a 284 bp mimic. Lane 1, 100 bp marker ladder; lane 2, 1 ng target; lane 3, 100 pg target; lane 4, 10 pg target; lane 5, 1 pg target; lane 6, 100 fg target; lane 7, negative control; lane 8, 304 bp target control (10 pg); lane 9, 284 bp mimic control (10 pg). The 304 bp target was competitively amplified in the presence of 10 pg mimic.

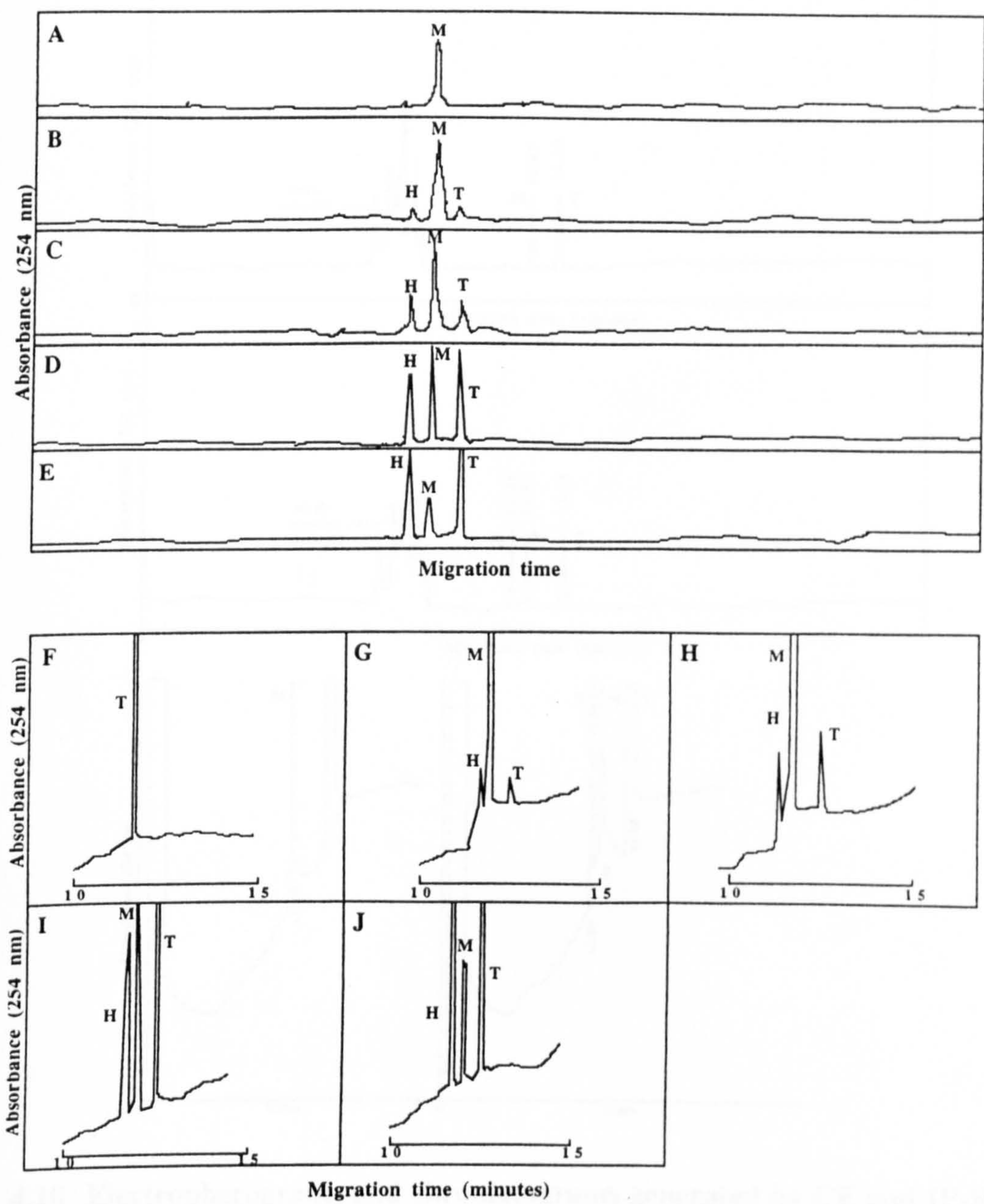


Figure 4.9 Products from competitive PCR using a 284 bp mimic analysed by CE and IP-RP-HPLC. Electropherograms A to E represent product obtained from sequential reactions containing a fixed amount of mimic co-amplified in the presence of decreasing amounts of target. Chromatograms F to J show the corresponding data obtained using IP-RP-HPLC. Peaks represent absorbance at 254 nm of the following products: **T** denotes the target product, **M** mimic product and **H** heteroduplex product

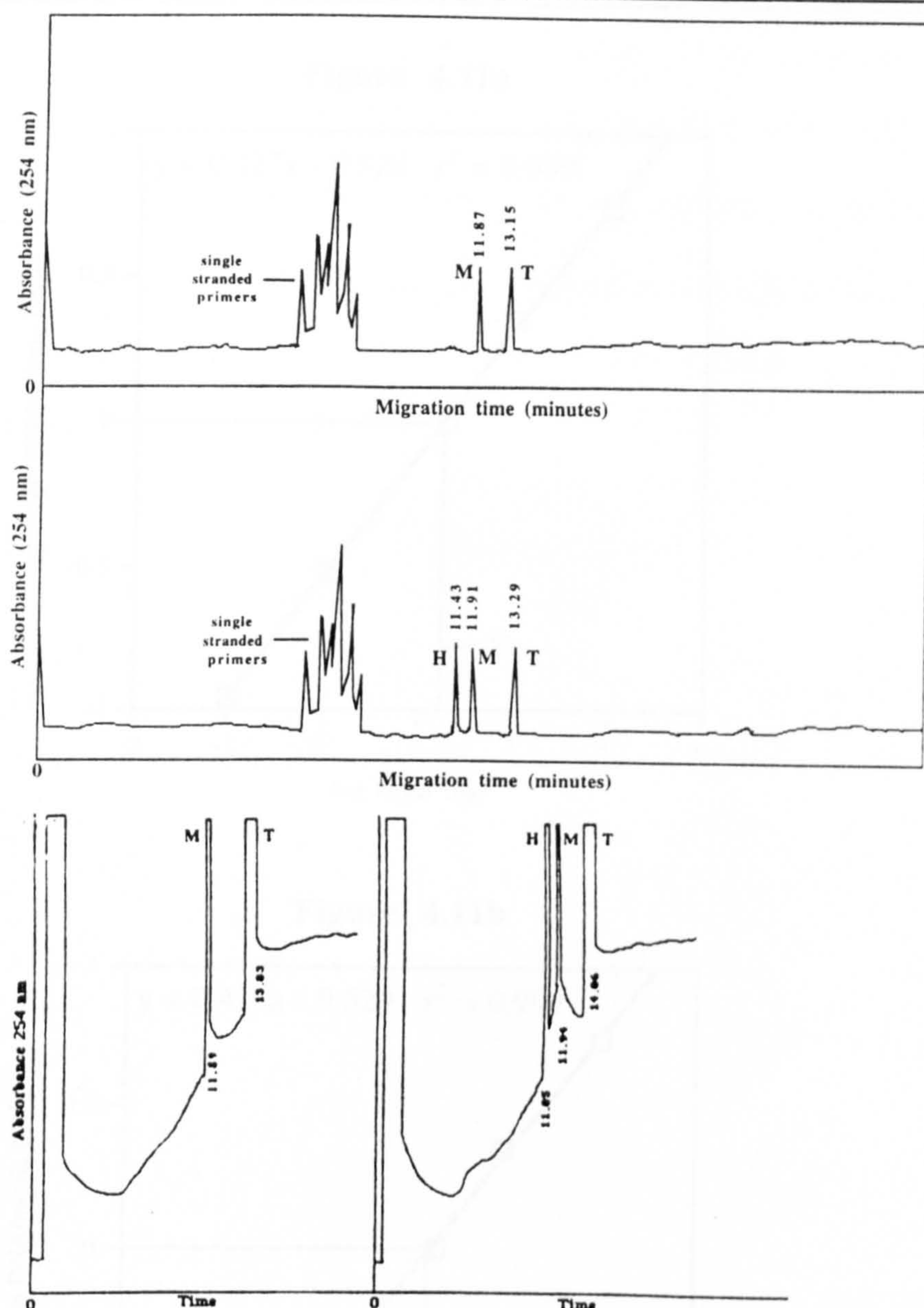


Figure 4.10 Electropherograms and chromatograms generated by CE and IP-RP-HPLC analysis of competitive PCR products illustrating the formation of heteroduplex products. The electropherograms were produced on CE analysis of an aliquot (1 μ l) of a mixture of products from two separate PCRs, one generating only target product, the other only mimic product. The bottom electropherogram was produced by analysis of the same mixture (1 μ l), heated to 94°C and then cooled to 4°C, resulting in the formation of a third peak (heteroduplex). The chromatograms were produced on IP-RP-HPLC analysis of a 10 μ l aliquot of mixed PCR products. The right-hand chromatogram showing the formation of heteroduplex product was generated using an aliquot of the same mixture (10 μ l). Peaks represent absorbance at 254 nm of the following products: **T** denotes the target product, **M** mimic product and **H** heteroduplex product, elution times are indicated.

Figure 4.11a

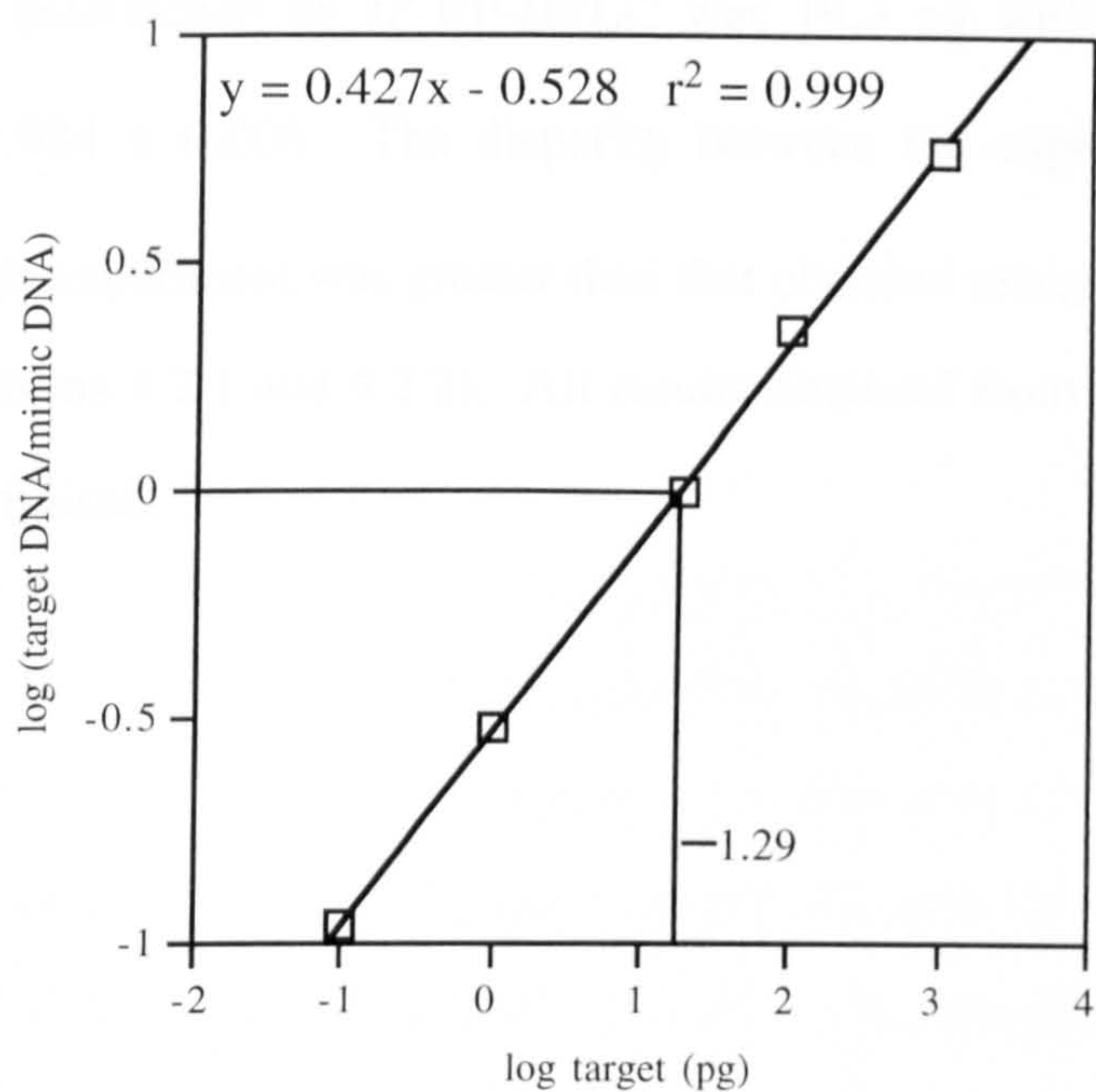


Figure 4.11b

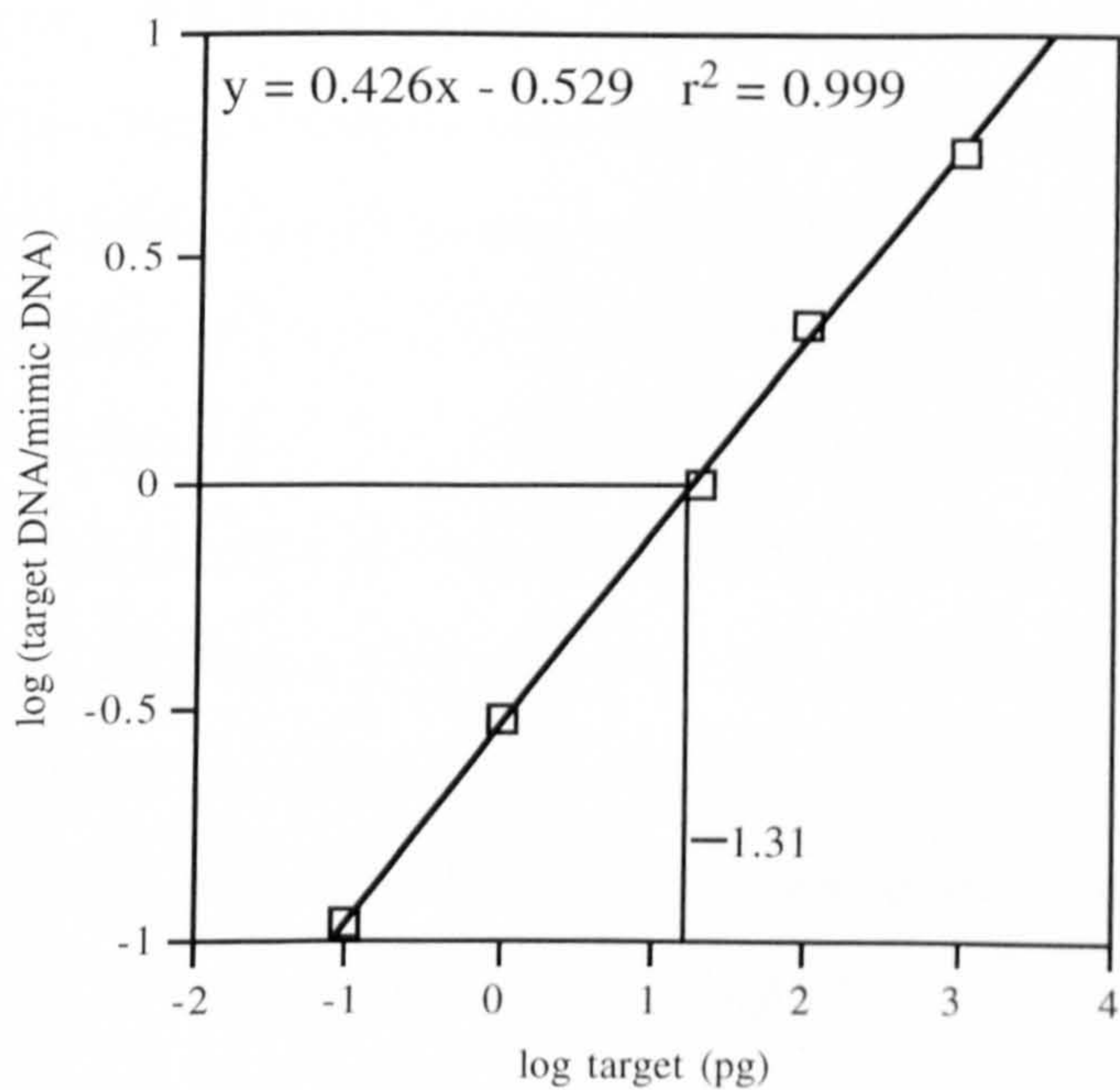


Figure 4.11 Determination of target concentration by competitive PCR using a 284 bp mimic following CE and IP-RP-HPLC analysis. Figure 4.11a shows the regression plot generated by a representative competitive PCR titration reaction using data obtained by CE analysis. Figure 4.11b shows a similar representative plot obtained by IP-RP-HPLC analysis of competitive PCR products.

with a mean coefficient of variation of 0.989 ± 0.005 . The mean initial target concentration determined by IP-RP-HPLC was 19.3 pg with a mean coefficient of variation of 0.984 ± 0.006 . The disparity between the expected and actual results obtained for this experiment was greater than that obtained using a 900 bp target and 304 bp mimic (Sections 4.2.1 and 4.2.2). All results obtained from these experiments were performed in triplicate.

4.3 Discussion

The analysis of DNA by slab gel electrophoresis gives relatively low levels of resolution in comparison to CE and HPLC and is generally unsatisfactory for the generation of accurate, quantitative data (Andrews, 1986). Furthermore, to detect amplification products, DNA must be stained with potentially toxic dyes, or reaction products must be labelled or hybridised to probes so as to visualise them by fluorescence, electrochemiluminescence, autoradiography or phosphor imaging. The use of CE and IP-RP-HPLC avoids these time-consuming and labour-intensive activities and also improves the accuracy of quantification by reduction of sample handling. Both the systems used allow PCR products to be analysed directly without the need for further sample handling. Quantification of reaction products using on-line UV absorbance detection provides accurate information concerning the amount of reaction product present in each peak.

The sharp, single product peaks obtained using both CE and IP-RP-HPLC indicate that both the mimic and target products were being synthesised to completion in the PCR as any incomplete fragments would be observed as either slopes on the left side of the peaks or as additional peaks.

Although certain precautions can be taken (as discussed in Section 3.3) to minimise the effect of sequence length and composition on the competitive amplification of a mimic (thus ensuring an equal amplification efficiency to that of a target) confirmation of equal amplification kinetics needs to be determined experimentally. In general, competitively amplified mimics and targets will eventually reach a limiting value as product ratios drift from a 1:1 molar ratio, beyond which the amplification kinetics are no longer similar enough to give reliable data (Innis and Gelfand, 1990). However, it is this equivalence point that needs to be ascertained for quantification purposes, thus the points on either side of this ratio are the critical points that must be demonstrated experimentally to be reliable.

We have shown here that the amount of DNA can be accurately determined using competitive PCR in conjunction with both CE and IP-RP-HPLC. Whilst the values of the initial target concentration obtained in Sections 4.2.1 and 4.2.2 should have been the same (10 pg) the data obtained using the *Hae* III/pGEM standards suggests the values determined by competitive PCR to be accurate and the discrepancy between the experimentally determined value and the anticipated value to result from errors with the original concentration determination and dilution. There is no reason to assume that the original estimation of DNA concentration or the dilution series prepared prior to amplification were any more accurate than the competitive PCR and CE or IP-RP-HPLC assays. The fact that the size of the competitor utilised for these experiments in Section 4.2 was significantly shorter than the target means it could potentially amplify more efficiently. This would lead to an observed over-estimation of the amount of competitor leading to an actual over-estimation of the amount of target at the equivalence point. This hypothesis fits the data obtained and could explain the apparent disparity between the observed and expected results.

The sensitivity and linearity of CE and IP-RP-HPLC analysis of PCR products adds greatly to the accuracy of the determination of mimic and target ratios. The observed linearity was acceptable with typical r^2 values of 0.999 or greater. Analyses typically require only small amounts of PCR products, which permits replication of quantitative analysis. To perform this type of analysis by HPLC is more cost-effective than other methods such as fluorescence or isotopic detection. Reduction in volume and optimisation of the elution profile for products within a defined size range would permit the analysis of up to 30 samples per hour with little operator participation beyond loading samples (Huber *et al.*, 1995). This effectively minimises an inherent limitation of HPLC, which is the necessity of sequential sample analysis.

However, CE offers a number of advantages over the techniques currently employed for the quantification of nucleic acids including IP-RP-HPLC. Only a fraction of the sample

is required (typically 1 µl of a PCR mixture) allowing a number of different analyses to be performed on the product, which is an important point when considering the rarity of some biological samples. The use of electrokinetic injection followed by data collection at 20 Hz leads to greater precision of injection and peak resolution by comparison with other systems, including IP-RP HPLC. This feature, combined with systems designed for high throughput automated analysis, would allow analysis with unrivalled speed and processivity allowing exceptional levels of accuracy and sensitivity for quantification by competitive PCR.

An additional advantage that emerged from the application of these two systems to competitive PCR was the ability to monitor heteroduplex formation using a target and mimic with very similar sequences. During the course of experiments, the formation of heteroduplexes during PCR was encountered in reactions employing a mimic which differed in length by 20 bases from that of the original target.

In this study, heteroduplexes were generated by random cross-hybridisation of the 304 bp target and 284 bp mimic sequences, which share approximately 62% homology. During the early cycles of PCR, the initial excess of primers ensures that once template DNA becomes denatured, it will anneal to primers rather than itself. Subsequent extension by *Taq* DNA polymerase generates homoduplexes (nucleic acid duplexes whose single strands are the precise complement of one another). As the polymerisation reaction proceeds through exponential amplification and approaches plateau, the ratio of template to primer increases dramatically with each cycle. This excess of product results in single template strands annealing to themselves in preference to primers and, amongst other parameters, is associated with the dramatic reduction in amplification efficiency observed during the later cycles of PCR. When more than one PCR product is present in the amplification reaction, template-template annealing at the later stages of PCR results in the formation of homoduplexes (self-annealing of complementary strands to generate target-target and mimic-mimic duplexes). In situations where both product species share

a high degree of sequence homology, heteroduplexes are formed (non-specific annealing of dissociated strands to generate target-mimic and mimic-target duplexes). Amplification of both the 304 bp and 284 bp DNA during the early to mid-exponential stages of PCR results in the generation of homoduplex species. However, as the polymerisation reaction enters the late exponential and plateau stages, the vast excess of template strands over primer molecules drives the reaction towards template-template strand reassortment and heteroduplex formation, rather than primer annealing and extension to generate more homoduplexes. Heteroduplexes are formed at the expense of homoduplex amplification, as the amount of heteroduplexes increases the total amount of homoduplex species progressively decreases (Henley *et al.*, 1996). Strand reassortment during these later stages is associated with a dramatic reduction in the rate of amplification, until eventually the target molecules reach saturating levels. At this point, the efficiency of amplification approaches zero and repeated rounds of thermal cycling will only result in random strand reassortment. This is a strong argument against quantifying competitively amplified PCR products in the plateau phase, in particular for those detection methods that fail to resolve heteroduplex from amplified product.

Although heteroduplex formation appears to present a specific problem to PCR it must also be noted that just as many (and probably more) polymerase-resistant homoduplexes are formed; all of which will be denatured in the next cycle.

One other potential source of bias, resulting in differences between the actual and observed results obtained using competitive PCR, could arise from differences in the reannealing rates of the target and mimic homoduplexes. If, due to differences in T_m and/or length, the target and mimic formed homoduplexes at different rates then this could preferentially remove one from a PCR-amplifiable state. This applies even where heteroduplexes are not an issue.

Heteroduplex formation is common during competitive PCR employing highly homologous sample and internal standard targets, and has been reported by a number of investigators, including Apostolakos *et al.*, 1993; Becker-André and Hahlbrock, 1989; Hahn *et al.*, 1995; Hayward *et al.*, 1998; Henley *et al.*, 1996; Hoff *et al.*, 1999; Lu *et al.*, 1993; McCulloch *et al.*, 1995). A number of solutions for the reduction or prevention of heteroduplex formation have been reported in the literature. The simplest is to restrict the number of amplification cycles in order to generate quantitative data prior to the formation of heteroduplexes (Schneeberger *et al.*, 1995). However, as heteroduplex formation is dependent on the initial concentration of template, this approach is not a viable method of compensating for heteroduplex formation in routine quantitative PCR. Alternative preventative measures have been reported by Becker-André and Hahlbrock (1989), who demonstrate that an additional amplification cycle after dilution of the PCR products will eradicate heteroduplexes. However, this procedure reduces the total amount of PCR product generated, and further manipulations are required to restore sensitivity and enable the detection and subsequent quantification of amplicons, e.g., radioactive labelling, blotting and probe hybridisation. Other methods include the addition of labelled primers prior to the last amplification cycle: only those duplexes generated following labelled primer annealing and extension by *Taq* DNA polymerase would be detectable (Lu *et al.*, 1993).

The potential generation of heteroduplexes in competitive PCR leads to a decision whether to construct a mimic which differs in size from the DNA target or which differs in the presence of a selected restriction site, but not in overall size. Restriction enzymes are unable to digest heteroduplexes that contain recognition sites for the restriction enzyme on only one strand, and any undigested products of the same size as the target would be mistakenly added to the balance of the undigested reaction product used to calculate the final product ratio. This factor, combined with the inaccurate results achieved using a mimic and target differing greatly in size (Sections 4.2.1 and 4.2.2), led to the construction of a mimic, which differed from the original target by 20 bp.

Determination of the initial concentration of a 304 bp target, co-amplified with this mimic, using both techniques showed a marked difference between the observed and expected results (Figure 4.11). Furthermore, the formation of heteroduplexes was apparent through the appearance of an additional product in both CE electropherograms and IP-RP-HPLC chromatograms (Figure 4.10).

The migration of such a duplex using gel electrophoresis is unpredictable due to the effect on mobility of the flexible single-stranded portion of the duplex and quantification of the heteroduplex using ethidium bromide staining appears to be unreliable (Schneeberger *et al.*, 1995). The identity of the heteroduplex formed was confirmed experimentally by melting and annealing a mixture of pure homoduplexes of both the mimic and target products. Heteroduplexes were more rapidly digested by S1 nuclease than homoduplexes (Howard *et al.*, 1999). Figure 4.9 showed that heteroduplexes were not identified when products were analysed by conventional agarose gel electrophoresis. The presence of heteroduplex formation was a consequence of sequence similarity between the target and mimic products. However, such similarity is required in order for the amplification efficiency of both the target and mimic products to be equal. Ideally, the target and mimic sequences should be unrelated, except for the primer binding sites, and have similar amplification efficiencies.

The analysis of heteroduplex formation to the quantitative accuracy of competitive PCR can not be over emphasised. Initial starting concentrations are calculated from the ratio of the reaction products. Since heteroduplexes subtract in equimolar amounts from both homoduplex reaction products, by determining the amount of heteroduplex present in a reaction and reallocating each of its components back to the corresponding homoduplex, the error generated by heteroduplex formation could theoretically be corrected (Boer and Ramamoorthy, 1997). This investigation demonstrates the ease with which heteroduplexes can form between a target and a mimic. However, both CE and IP-RP-HPLC analysis could potentially be used to correct for the formation of these products.

Raeymakers, (1993) has analysed some of the mathematical considerations concerning competitive PCR and concluded that a plot of log product ratio against log of the initial amount of mimic added to the PCR should be linear with a slope of unity. As demonstrated in Figures 4.4 and 4.8, the concentration of PCR products and target conformed to this linear relationship with slope close to unity. A similar result was generated using a 304 bp target (and 284 bp mimic) following reallocation of heteroduplexes back to its original homoduplex (Figure 4.11). This appears to be an important advantage of these techniques when compared to other methods that have generated slope values greatly different from unity (Gilliland *et al.*, 1990; Piatak *et al.*, 1993; Siebert and Larrick, 1992).

CHAPTER 5

QUANTIFICATION OF *LEGIONELLA* *PNEUMOPHILA* BY CONTINUOUS FLUORESCENCE MONITORING DURING AMPLIFICATION

5. QUANTIFICATION OF *LEGIONELLA PNEUMOPHILA* BY CONTINUOUS FLUORESCENCE MONITORING DURING AMPLIFICATION

5.1 Introduction

The quantification of nucleic acids by PCR is complex because the efficiency of amplification depends on a large number of variables (discussed in Section 3.3). Therefore, the initial amount of a target template can only be calculated from the final, amplified product yield if the amplification efficiency has been determined or variations in amplification efficiency are controlled for by the addition of a competitive standard. To determine accurately the efficiency of amplification during the exponential phase, the kinetics of the reaction have to be analysed either by serial dilution of the target DNA in the PCR or by removing a portion of the reaction after successive cycles (Dupl  a *et al.*, 1993; Murphy *et al.*, 1990). Since the exponential range for each sample analysed should be empirically determined these methods become very laborious indeed (Murphy *et al.*, 1990). As a result, competitive PCR has become the method of choice for quantification because, due to the competitive nature of the assay, there is no need to restrict amplification to a narrow range of the exponential phase. However, competitive PCR is not without problems, and as a consequence, it has been suggested that the amplification efficiencies of both the target and competitor should be carefully monitored at all stages of the reaction to ensure equal amplification efficiencies (Becker-Andr   and Hahlbrock, 1989).

The work presented in Chapter 3, demonstrated that the length and composition of target and mimic sequences could have a profound effect on their amplification efficiencies in a competitive reaction. Furthermore, gel analysis of competitive PCR products proved difficult and time consuming. Attempts to quantify DNA using mimics of similar and

very different sizes, by CE and IP-RP-HPLC, revealed problems associated with each of these particular approaches (discussed in Section 4.3). Furthermore the protocols used were hampered by two common features (*i*) iterations were required to ensure the samples were within the dynamic range for accurate quantification and (*ii*) the methods were labour-intensive. Although it is often possible to complete such a PCR-based analysis within a single working day, there is still a need to reduce the analysis time still further. Both CE and IP-RP-HPLC suffer the additional disadvantage that two steps are necessary to measure reaction products. The ideal approach would reduce post-PCR analysis to a single step, eliminating the problems associated with conventional forms of analysis, lend itself to automation and produce accurate and reproducible measurements of initial target concentration.

The recent development of integrated systems such as the LightCyclerTM and the ABI PRISM[®] 7700 Sequence Detection System, for the simultaneous amplification and detection of PCR products, have begun to address these concerns because the amplification efficiencies of generated products can be monitored cycle by cycle, resulting in up to 8 logs of dynamic range in one PCR (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993).

The ABI PRISM[®] 7700 Sequence Detection System uses sequence-specific probes for detection. These probes use fluorescent dyes attached to target-specific oligonucleotides and allow the sensitive detection of specific target templates. However, a unique probe is required for each target and these probes can be difficult to manufacture. The technology itself is also very expensive. Alternatively, the LightCyclerTM uses a dsDNA dye which provides a cheaper and simpler general method for product detection however, quantification is difficult because all dsDNA products are detected using this system.

The aim of this particular study was therefore to develop low cost PCR based assays, using the LightCyclerTM, to assess the accuracy of quantification of *Legionella pneumophila* by continuous fluorescence monitoring.

5.2 Results

5.2.1 Melting curve analysis of competitive PCR products

To distinguish between the *Legionella pneumophila* target and competitive templates, the melting temperatures of the amplimers needed to be sufficiently different to separate the competitive and target components of the melting curve. The melting curves obtained for the 900 bp DNA target from *Legionella pneumophila* and the 304 bp mimic PCR products (44% GC) showed that their apparent melting temperatures differed by 2°C (Figure 5.1). This shift in melting temperature, caused by differences in GC content and length of the target and mimic, was enough to differentiate between products. A prerequisite for this type of assay is that both the target and mimic must amplify with equal amplification efficiencies (Ririe *et al.*, 1997). Determination of the amplification efficiencies of two mimics and the 900 bp target (Section 2.16.4.1) showed that at the ramp rates employed for the LightCycler™ (20°C s⁻¹), the target and mimics amplification efficiencies were the same. Furthermore, the efficiencies of amplification were similar to those obtained using conventional ramp rates (1°C s⁻¹) (Table 5.1).

Table 5.1 Amplification efficiencies of 304 bp mimics of varying % GC content and 900 bp target using selected ramp rates.

% GC content	Amplification efficiency (E) (Ramp rate = 20°C s ⁻¹)	Amplification efficiency (E) (Ramp rate = 1°C s ⁻¹)
304 bp mimic		
40	90%	93%
44	90%	92%
900 bp target		
53	90%	89%

Initially, melting curve analysis was performed on a competitive PCR containing a fixed amount of a 304 bp competitor mimic (10 pg) and a known dilution series of a 900 bp target ranging from 10 ng to 10 fg (Figures 5.2a and 5.2b). The equivalence point of the target and mimic could be observed as the melting curves generated during competitive

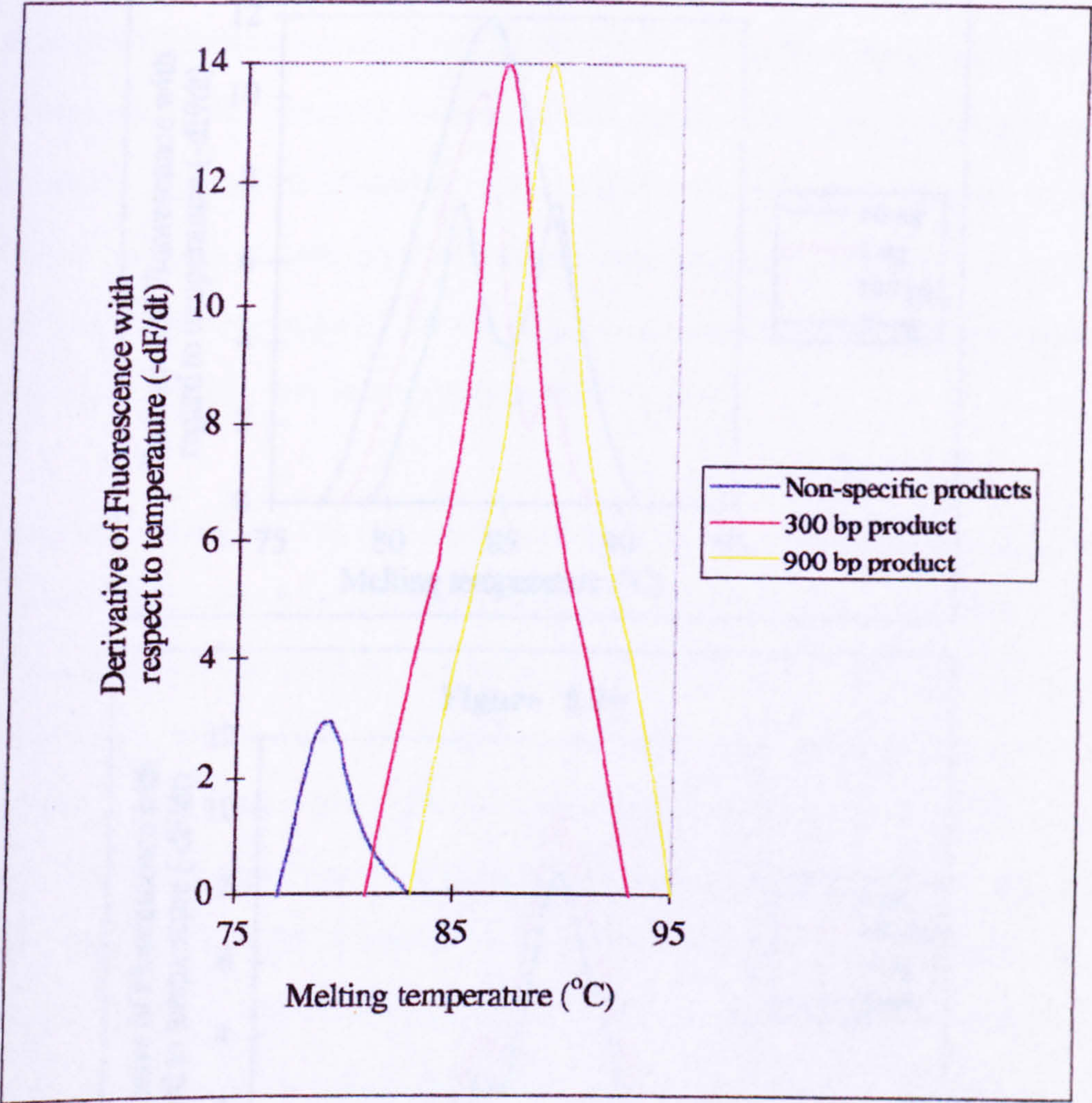


Figure 5.1 Determination of Melting temperature by Melting Curve Analysis. Melting curve peaks were obtained for a 900 bp PCR fragment (53% GC content) and a 304 bp fragment (44% GC content). Samples were amplified for 45 cycles using the thermal cycling conditions described in Section 2.16.4, prior to melting curve analysis.

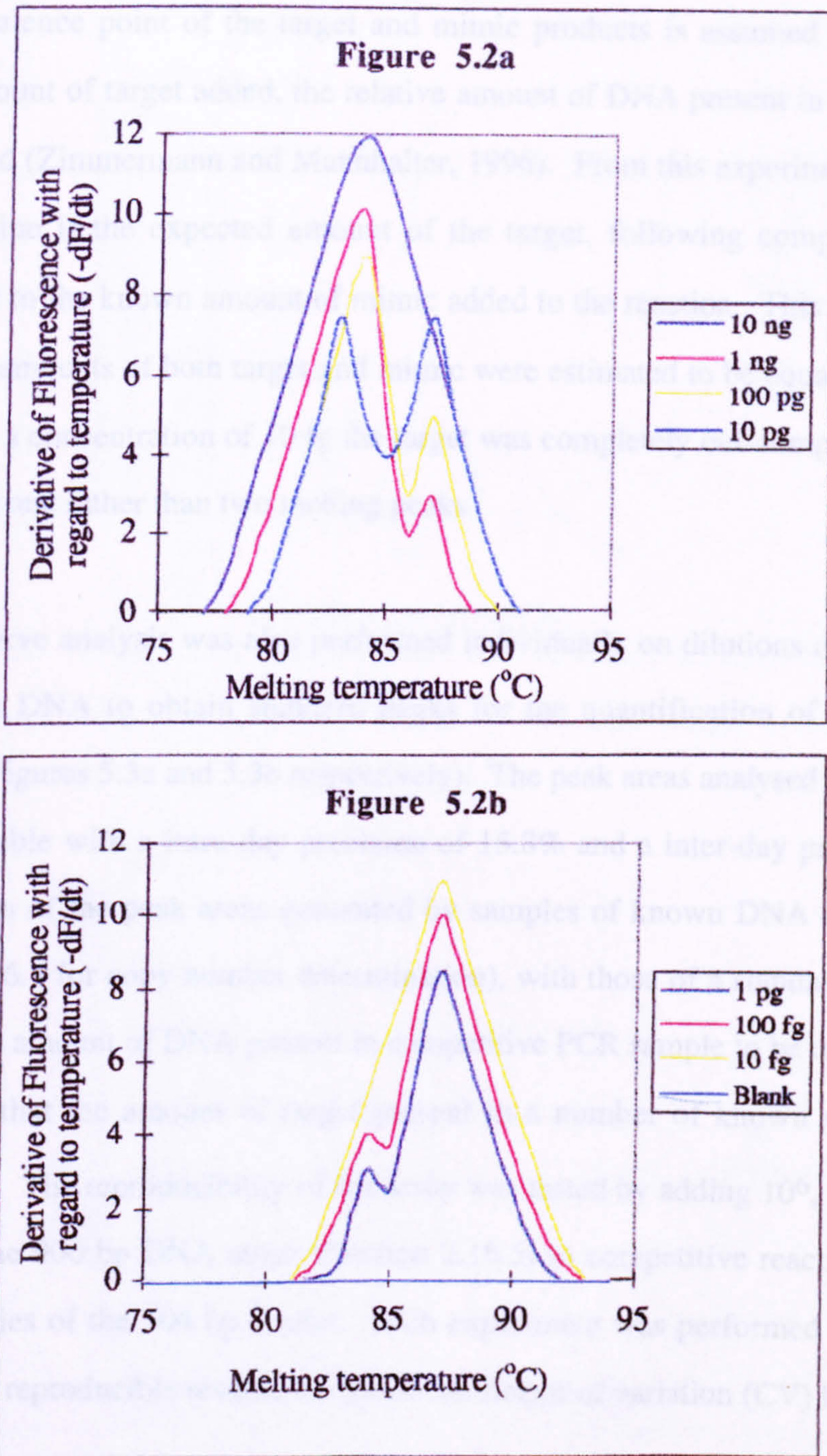


Figure 5.2 Melting curve analysis of competitive PCR products. Melting curve peaks were generated for a dilution series of a 900 bp target, ranging from 10 ng to 10 fg, competitively amplified in the presence of a fixed amount of a 304 bp mimic (10 pg) (Figures 5.2a and 5.2b). All samples were amplified for 45 cycles using standard conditions described in Section 2.16.4 before melting curve analysis.

Correction: In this figure the results used to generate the melting curves were transposed. The melting curves obtained for the 900 bp target, amplified in the presence of the 304 bp target, actually represent the amplified 304 bp product and *vice versa*.

PCR progress from mainly target to equal amounts of target and mimic product. Since the equivalence point of the target and mimic products is assumed to be equal to the initial amount of target added, the relative amount of DNA present in a sample could be ascertained (Zimmermann and Mannhalter, 1996). From this experiment it was possible to determine if the expected amount of the target, following competitive PCR, was equivalent to the known amount of mimic added to the reaction. This was confirmed, as the initial amounts of both target and mimic were estimated to be equal at approximately 10 pg. At a concentration of 10 fg the target was completely out-competed by the mimic generating one rather than two melting peaks.

Melting curve analysis was also performed individually on dilutions of amplified target and mimic DNA to obtain standard peaks for the quantification of competitive PCR products (Figures 5.3a and 5.3b respectively). The peak areas analysed were reproducible and repeatable with a intra-day precision of 15.8% and a inter-day precision of 24.3%. Comparison of the peak areas generated by samples of known DNA copy number (see Section 2.16.5 for copy number determination), with those of a standard mimic dilution, allowed the amount of DNA present in competitive PCR sample to be determined. Table 5.2 shows that the amount of target present in a number of known samples could be ascertained. The reproducibility of the assay was tested by adding 10^6 , 10^4 , or 10^2 initial copies of the 900 bp DNA target (Section 2.16.5) to competitive reactions containing a dilution series of the 304 bp mimic. Each experiment was performed on separate days and yielded reproducible results, the mean coefficient of variation (CV) being 4.5%.

The assay was repeated directly amplifying samples containing known numbers of *Legionella pneumophila* cells (Table 5.3). The mean cell numbers obtained from these samples accurately reflected the initial cell numbers added to the reactions.

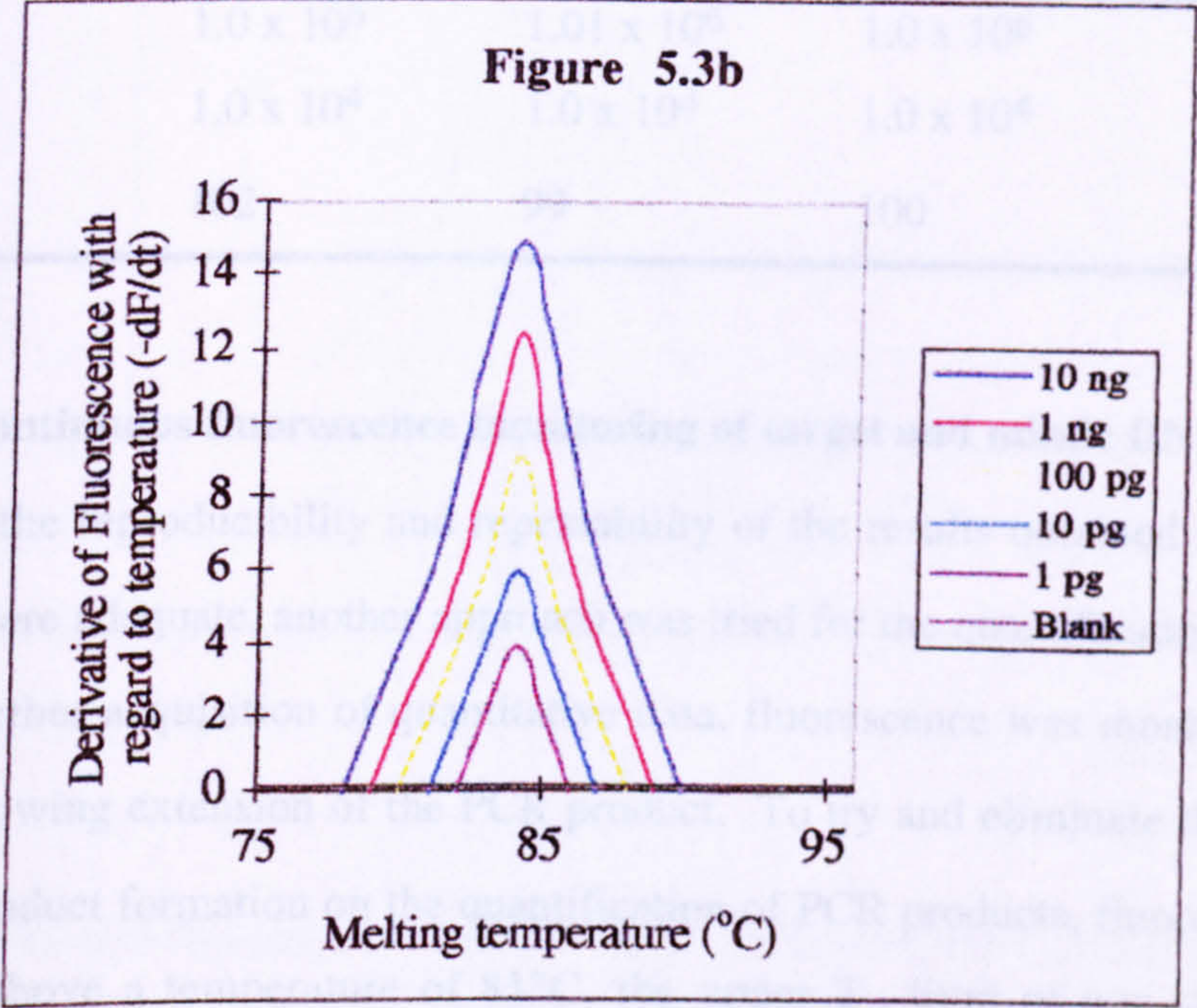
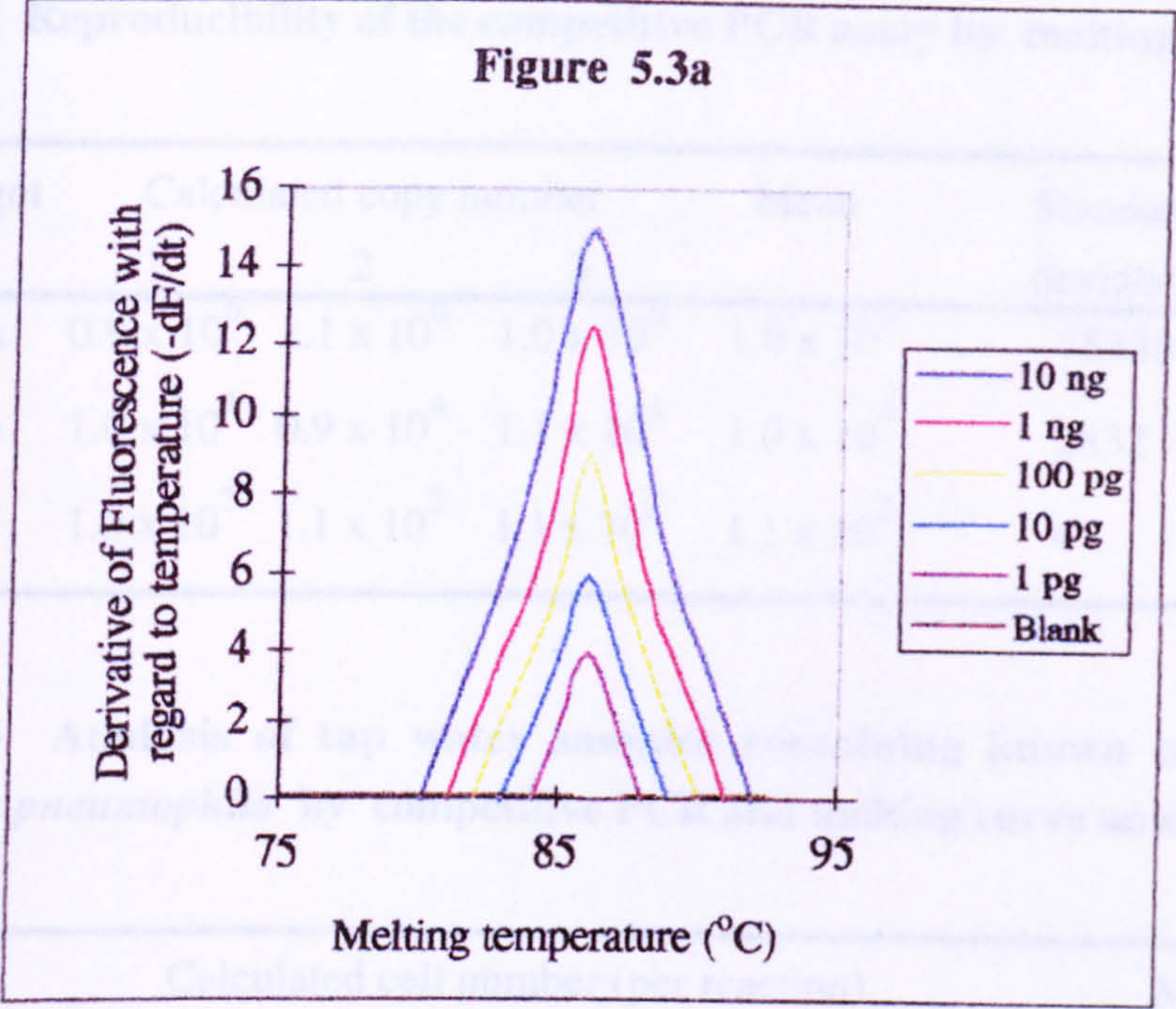


Figure 5.3 Standard peaks generated for a 900 bp target and 304 bp mimic by melting curve analysis. Dilutions of a 900 bp target (Figure 5.3a) and a 304 bp mimic (Figure 5.3b), ranging from 10 ng to 1 pg, were amplified for 45 cycles using standard methodology described in Section 2.16.4 before melting curve analysis.

Table 5.2 Reproducibility of the competitive PCR assay by melting curve analysis.

Initial target amount	Calculated copy number			Mean	Standard deviation	CV
	1	2	3			
10 ⁶ copies	0.9 x 10 ⁶	1.1 x 10 ⁶	1.0 x 10 ⁶	1.0 x 10 ⁶	75358	7.2%
10 ⁴ copies	1.0 x 10 ⁴	0.9 x 10 ⁴	1.1 x 10 ⁴	1.0 x 10 ⁴	2832	8%
10 ² copies	1.0 x 10 ²	1.1 x 10 ²	1.1 x 10 ²	1.1 x 10 ²	4	3.6%

Table 5.3 Analysis of tap water samples containing known cell numbers of *Legionella pneumophila* by competitive PCR and melting curve analysis.

Initial cell number	Calculated cell number (per reaction)			Mean
	1	2	3	
10 ⁶ cells	1.0 x 10 ⁶	1.01 x 10 ⁶	1.0 x 10 ⁶	1.0 x 10 ⁶
10 ⁴ cells	1.0 x 10 ⁴	1.0 x 10 ⁴	1.0 x 10 ⁴	1.0 x 10 ⁴
10 ² cells	102	99	100	100

5.2.2 Continuous fluorescence monitoring of target and mimic DNA

Although the reproducibility and repeatability of the results obtained by melting curve analysis were adequate, another approach was tried for the quantification of target DNA. For the further acquisition of quantitative data, fluorescence was monitored once every cycle, following extension of the PCR product. To try and eliminate the effect of non-specific product formation on the quantification of PCR products, fluorescence data was collected above a temperature of 81°C, the upper T_m limit of non-specific products. Target and mimic samples of varying initial template concentrations were amplified for 45 cycles. Since the log-linear increase above background fluorescence for both the target and mimic starts at a cycle number dependent on the initial starting concentration, fluorescence profiles obtained from known amounts of standard samples can be used to generate a standard graph for the quantification of unknown samples. Figure 5.4 shows a typical example of a standard graph following the subtraction of background

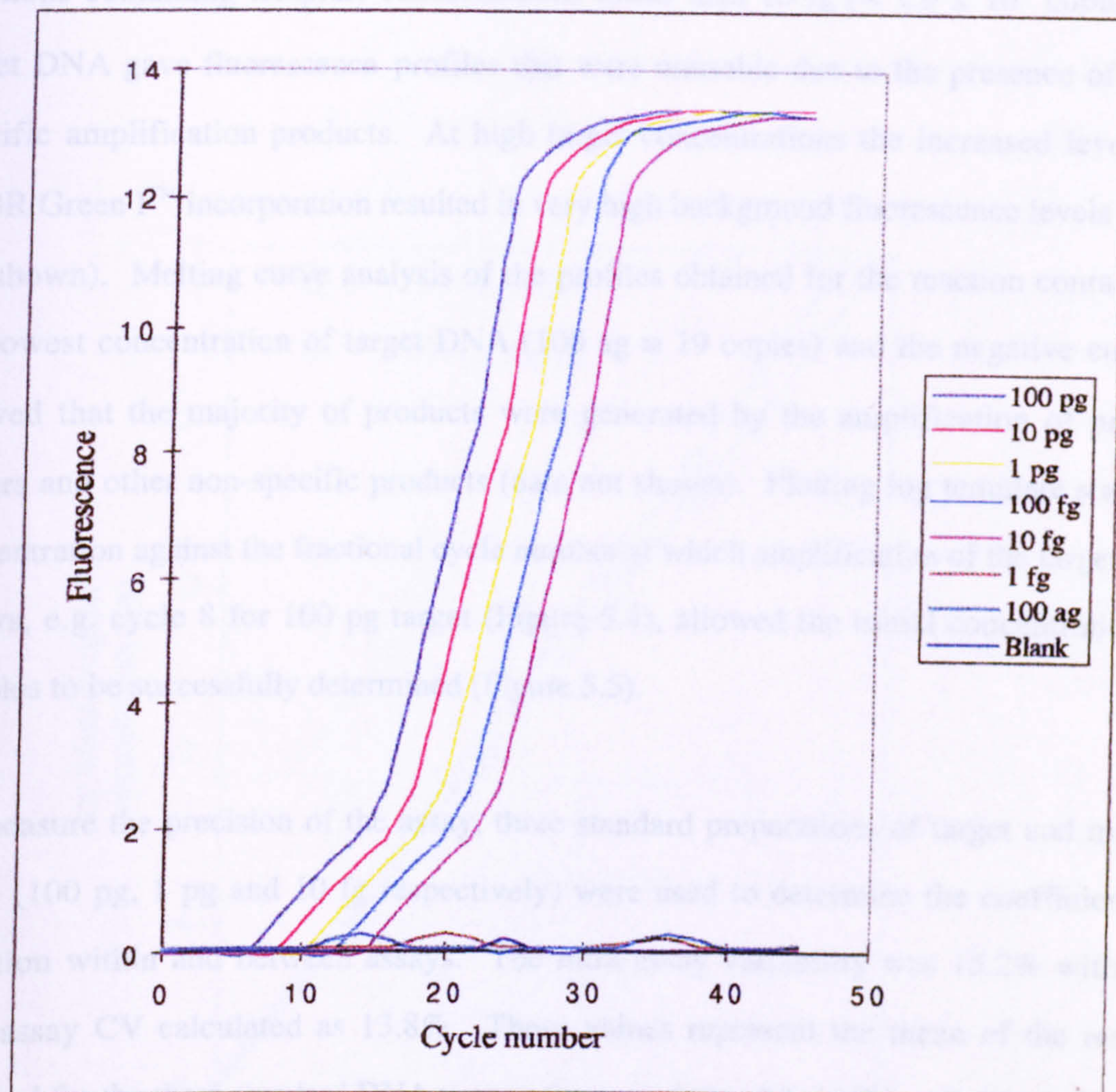


Figure 5.4 On-line fluorescence monitoring of PCR products. A dilution series of a 900 bp target was amplified for 45 cycles to generate standard fluorescence profiles for the production of a standard graph. The initial template concentrations ranged from 100 pg to 100 ag. The thermal cycling conditions used for amplification were as described in Section 2.16.4. The fluorescence profiles were separated by two amplification cycles.

fluorescence. Reactions containing initial template concentrations ranging from 100 pg to 10 fg generated standard profiles. However, melting curve analysis showed those reactions containing template concentrations lower than 10 fg ($\equiv 1.9 \times 10^3$ copies) of target DNA gave fluorescence profiles that were unusable due to the presence of non-specific amplification products. At high target concentrations the increased levels of SYBR Green ITM incorporation resulted in very high background fluorescence levels (data not shown). Melting curve analysis of the profiles obtained for the reaction containing the lowest concentration of target DNA (100 ag \equiv 19 copies) and the negative control showed that the majority of products were generated by the amplification of primer dimers and other non-specific products (data not shown). Plotting log template starting concentration against the fractional cycle number at which amplification of the target first occurs, e.g. cycle 8 for 100 pg target (Figure 5.4), allowed the initial concentration of samples to be successfully determined (Figure 5.5).

To measure the precision of the assay, three standard preparations of target and mimic DNA (100 pg, 1 pg and 10 fg respectively) were used to determine the coefficient of variation within and between assays. The intra-assay variability was 15.2% with the inter-assay CV calculated as 13.8%. These values represent the mean of the results obtained for the three standard DNA preparations, performed in triplicate at three separate times. Both intra-assay and inter-assay variability were lowest at standard concentrations of 100 pg and 1 pg. The CVs obtained using 10 fg of both target and mimic DNA were considerably larger, reflecting the lack of specific PCR products generated at low template concentrations (Table 5.4).

Analysis of tap water samples containing known cell numbers of *Legionella pneumophila* using this standard graph methodology, confirmed the validity of this assay for the quantification of bacteria. Figure 5.6 shows the standard graph obtained for reactions containing initial cell numbers ranging from 1 to 10^5 cells per reaction. From the standard

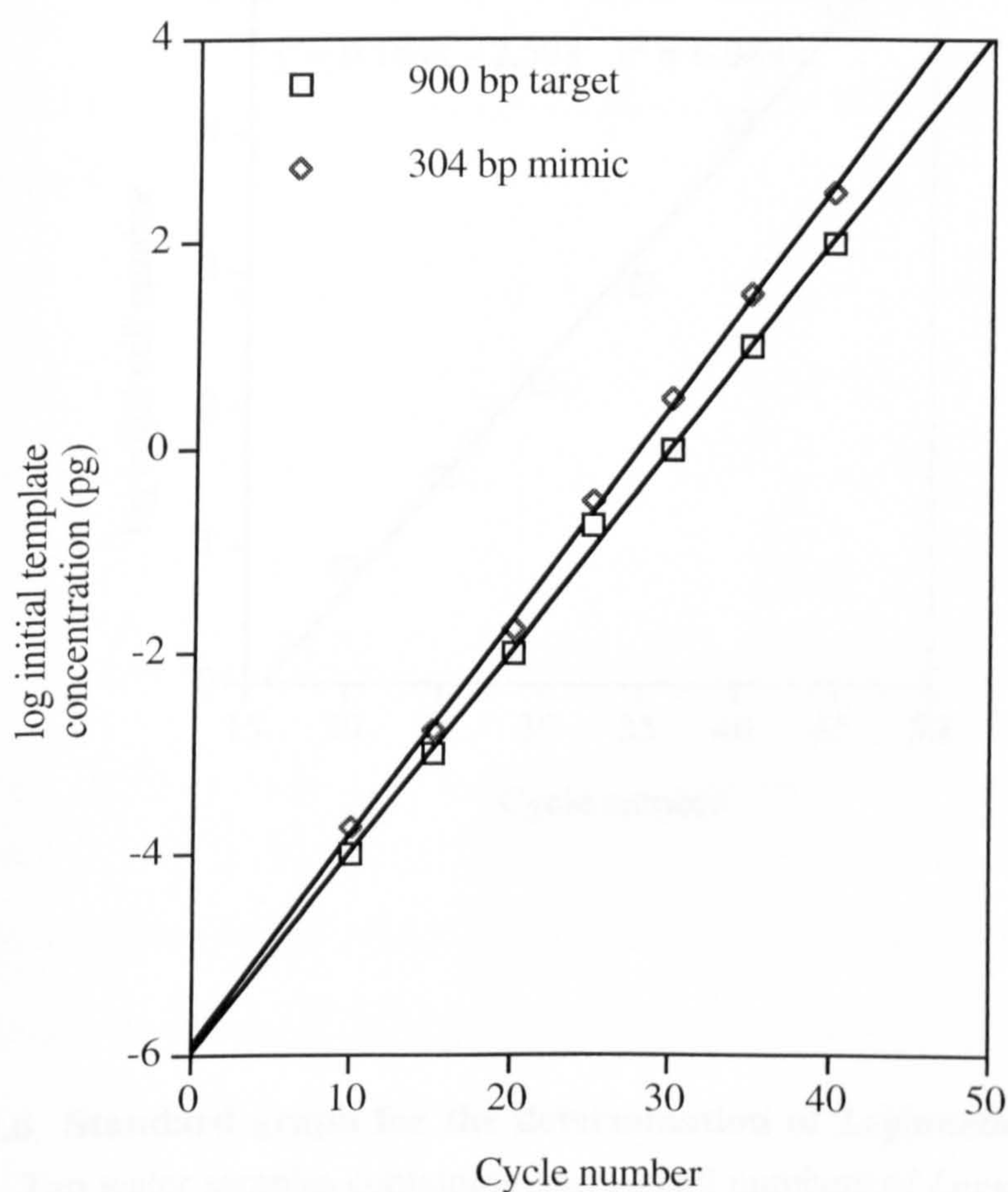


Figure 5.5 Standard graph for the quantification of initial target and mimic concentrations. The log of the template starting concentration was plotted against the fractional cycle number at fluorescence threshold. Linear relationships were obtained for plots of both the 900 bp target and the 304 bp mimic (r^2 values were 0.984 and 0.985 respectively).

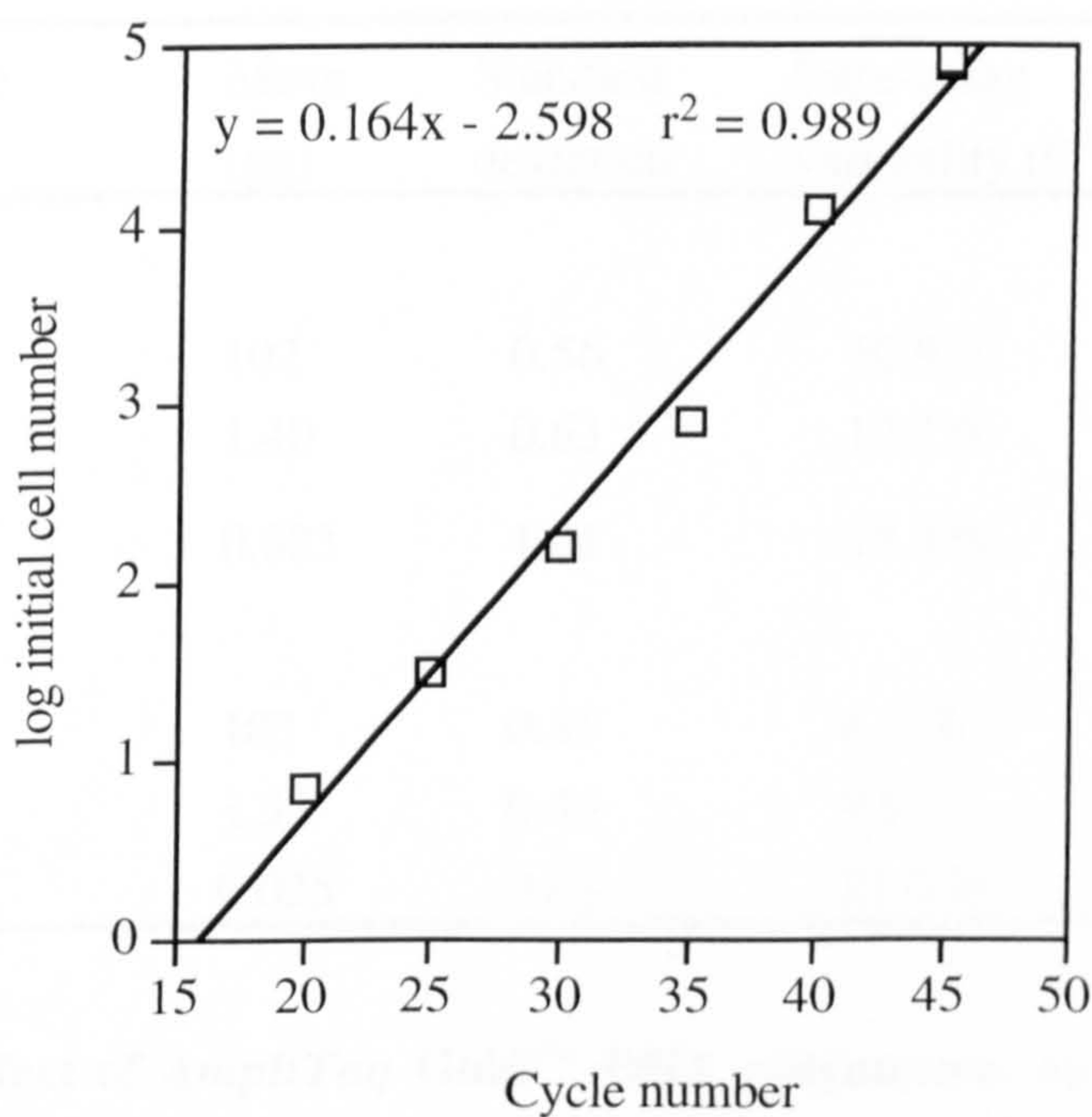


Figure 5.6 Standard graph for the determination of *Legionella pneumophila* cell number. Tap water samples containing known cell numbers of *Legionella pneumophila* were amplified for 45 cycles using the thermal cycling conditions described in Section 2.16.4. The fluorescence profiles obtained for these samples (initial cell numbers ranged from 1 to 10⁵ cells per PCR) were used to generate a standard graph for the quantification of *Legionella pneumophila*. The fluorescence profiles were separated by two amplification cycles.

graph it was possible to detect 10 cells of *Legionella pneumophila*. However, due to the lack of signal generated, it was not possible to detect bacteria below this value.

Table 5.4 Intra-assay and inter-assay variability of once-per-cycle fluorescence monitoring of target and mimic standard preparations.

Initial template concentration	Mean (pg)	Standard deviation	Intra-assay variability (CV)	Inter-assay variability (CV)
304 bp mimic				
100 pg	102	0.56	10.9 %	11.1%
1 pg	1.40	0.63	12.2 %	12.9%
0.01 pg	0.023	1.01	18.4 %	21.7%
900 bp target				
100 pg	103	0.31	6.3 %	7.8%
1 pg	1.35	0.49	9.9 %	10.6%
0.01 pg	0.025	0.83	21.0 %	23.1%

5.2.3 The effect of *AmpliTaq Gold*[™] DNA polymerase on non-specific product formation

To minimise the effect of primer dimer and non-specific product formation on the amplification of low copy number targets, *AmpliTaq Gold*[™] DNA polymerase was substituted for *Taq* DNA polymerase in reactions. This enzyme, upon activation by a pre-PCR heat step, prevents the non-specific annealing and extension of products that can occur when setting up reactions at room temperature. The subsequent amplification of non-specific products and primer dimers is therefore avoided in later PCR cycles. Figure 5.7a shows the fluorescence profiles obtained using a dilution series of a 900 bp target amplified for 45 cycles using *Taq* DNA polymerase. The reaction containing 1 pg target showed a decrease in slope, and examination of the melting curve revealed the formation of non-specific products (Figure 5.7b). At target concentrations below this value the formation of non-specific products predominated with little, if any target products being generated. Substituting *AmpliTaq Gold*[™] in the reaction mix increased the specificity of

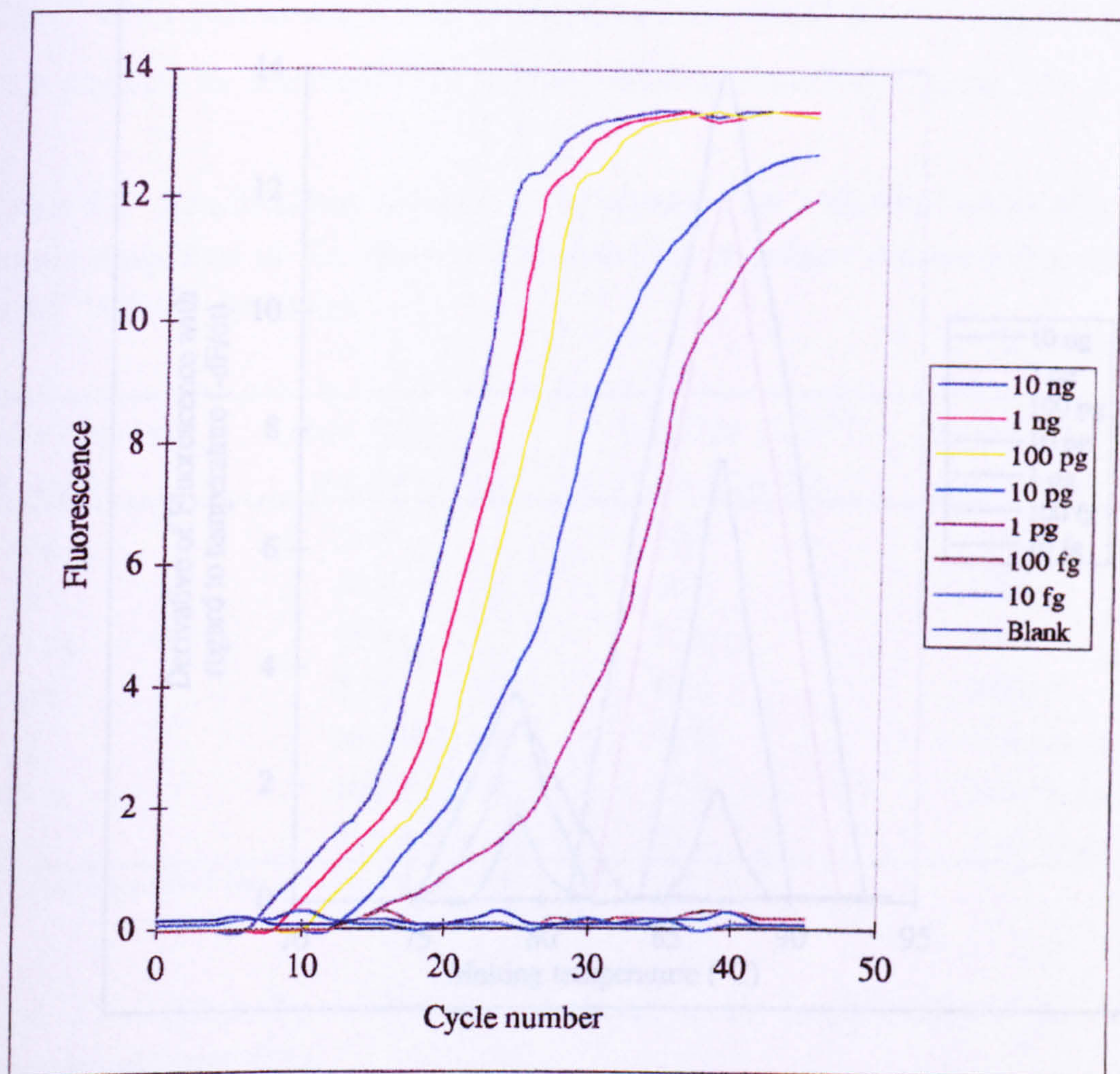


Figure 5.7a Fluorescence profiles generated for a dilution series of a 900 bp target using *Taq* DNA polymerase. Amounts of template DNA ranging from 10 ng to 10 pg were seen to amplify with similar efficiencies. (based on the raw data obtained from the fluorescence profiles generated). The amplification efficiencies of target dilution's below 10 pg (1 pg-10 fg) were seen to decrease with a reduction in template concentration. Samples were amplified for 45 cycles using the thermal cycling conditions described in Section 2.16.4, prior to melting curve analysis.

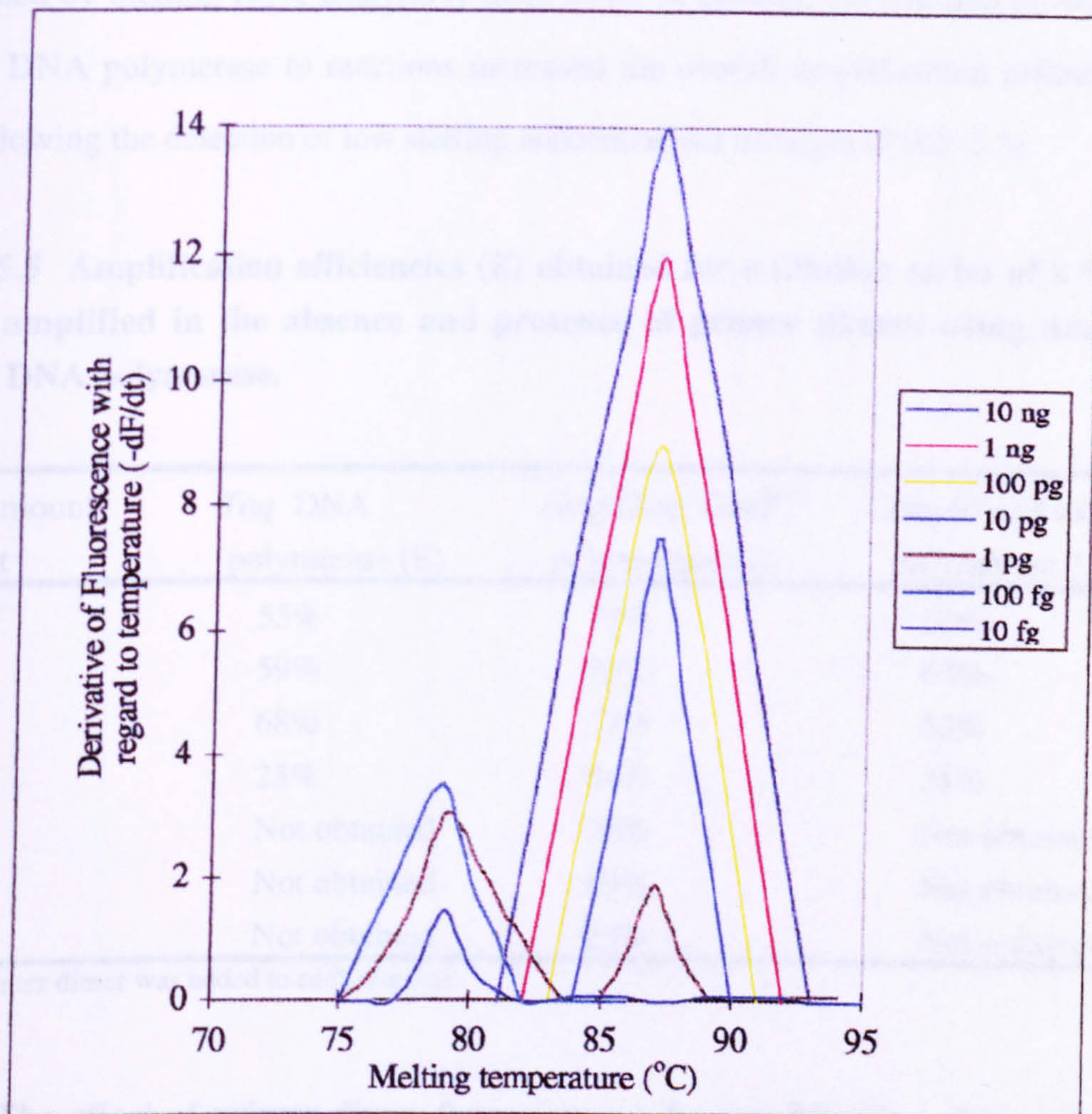


Figure 5.7b Melting curve analysis of PCR products generated from the amplification of a dilution series of a 900 bp target using *Taq* DNA polymerase. Melting peaks obtained following the amplification of 10 ng, 1 ng, 100 pg and 10 pg template DNA confirmed the homogeneity of the products generated. Initial starting concentrations of template ranging from 1 pg-10 fg generated peak profiles indicative of non-specific amplification products. Melting curves were obtained after 30 seconds of annealing at 74°C, followed by a 0.1°C s⁻¹ linear temperature transition to 94°C.

the PCR conditions allowing the amplification of specific products from template concentrations as low as 100 fg (Figure 5.8a). The purity of the PCR products was confirmed by melting curve analysis (Figure 5.8b). In general, the addition of *AmpliTaq Gold*TM DNA polymerase to reactions increased the overall amplification efficiency of PCR allowing the detection of low starting concentrations of target (Table 5.5).

Table 5.5 Amplification efficiencies (E) obtained for a dilution series of a 900 bp target amplified in the absence and presence of primer dimers using *AmpliTaq Gold*TM DNA polymerase.

Initial amount of target	<i>Taq</i> DNA polymerase (E)	<i>AmpliTaq Gold</i> TM polymerase (E)	<i>AmpliTaq Gold</i> TM polymerase * (E)
10 ng	55%	78%	60%
1 ng	59%	85%	64%
100 pg	68%	73%	53%
10 pg	25%	84%	31%
1 pg	Not obtained	75%	Not obtained
100 fg	Not obtained	69%	Not obtained
10 fg	Not obtained	23%	Not obtained

*10 fg primer dimer was added to each reaction

5.2.4 The effect of primer dimer formation on the amplification of low starting amounts of target DNA

In order to assess the effect of primer dimer formation on the amplification of low initial template concentrations further, a primer dimer (consisting of overlapping Leg primer sequences) was synthesised and 10 fg added to reactions. The presence of this primer dimer during amplification reversed the effect of *AmpliTaq Gold*TM, decreasing the specificity of the PCR conditions overall (Figure 5.9a). Non-specific products started to form at an initial template concentration of 10 pg, rather than at 100 fg, as seen in the previous experiment (Figure 5.9b). This was reflected in the amplification efficiencies obtained for this experiment (Table 5.5).

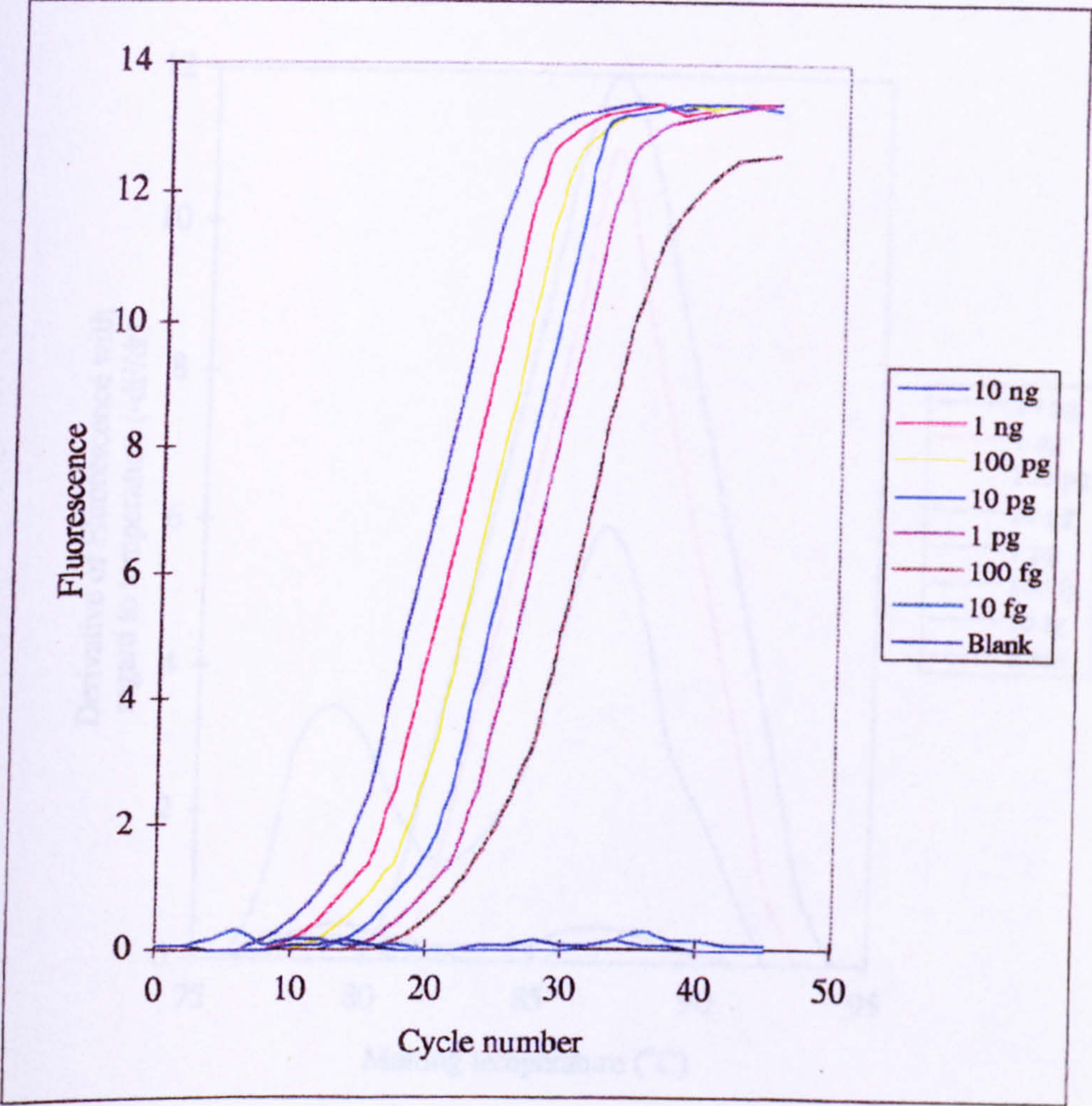


Figure 5.8a Fluorescence profiles generated for a dilution series of a 900 bp target using *AmpliTaq Gold*TM DNA polymerase. The amplification efficiencies of the target dilution's were similar over the range 10 ng-100 fg. A decrease in amplification efficiency was observed when an initial template concentration of 10 fg was used. Samples were amplified for 45 cycles using the thermal cycling conditions described in Section 2.16.4, prior to melting curve analysis.

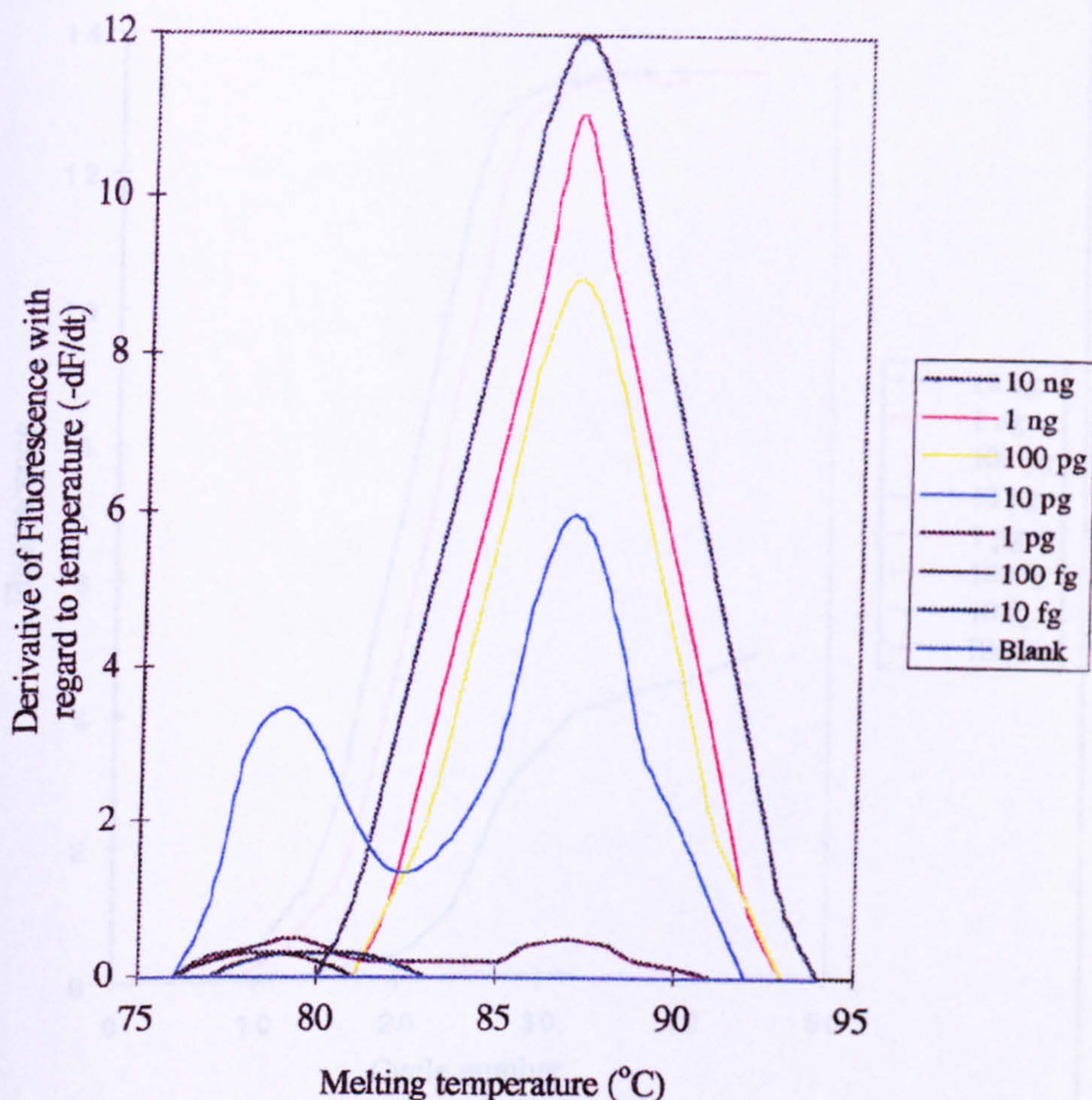


Figure 5.8b Melting curve analysis of PCR products generated from the amplification of a dilution series of a 900 bp target using *AmpliTaq Gold*[™] DNA polymerase. Analysis of the melting peaks confirmed the purity of the products generated using template concentrations ranging from 10 ng-100 fg. Non-specific amplification products were observed when a starting concentration of 10 fg was used. Melting curves were obtained after 30 seconds of annealing at 74°C, followed by a 0.1°C s⁻¹ linear temperature transition to 94°C.

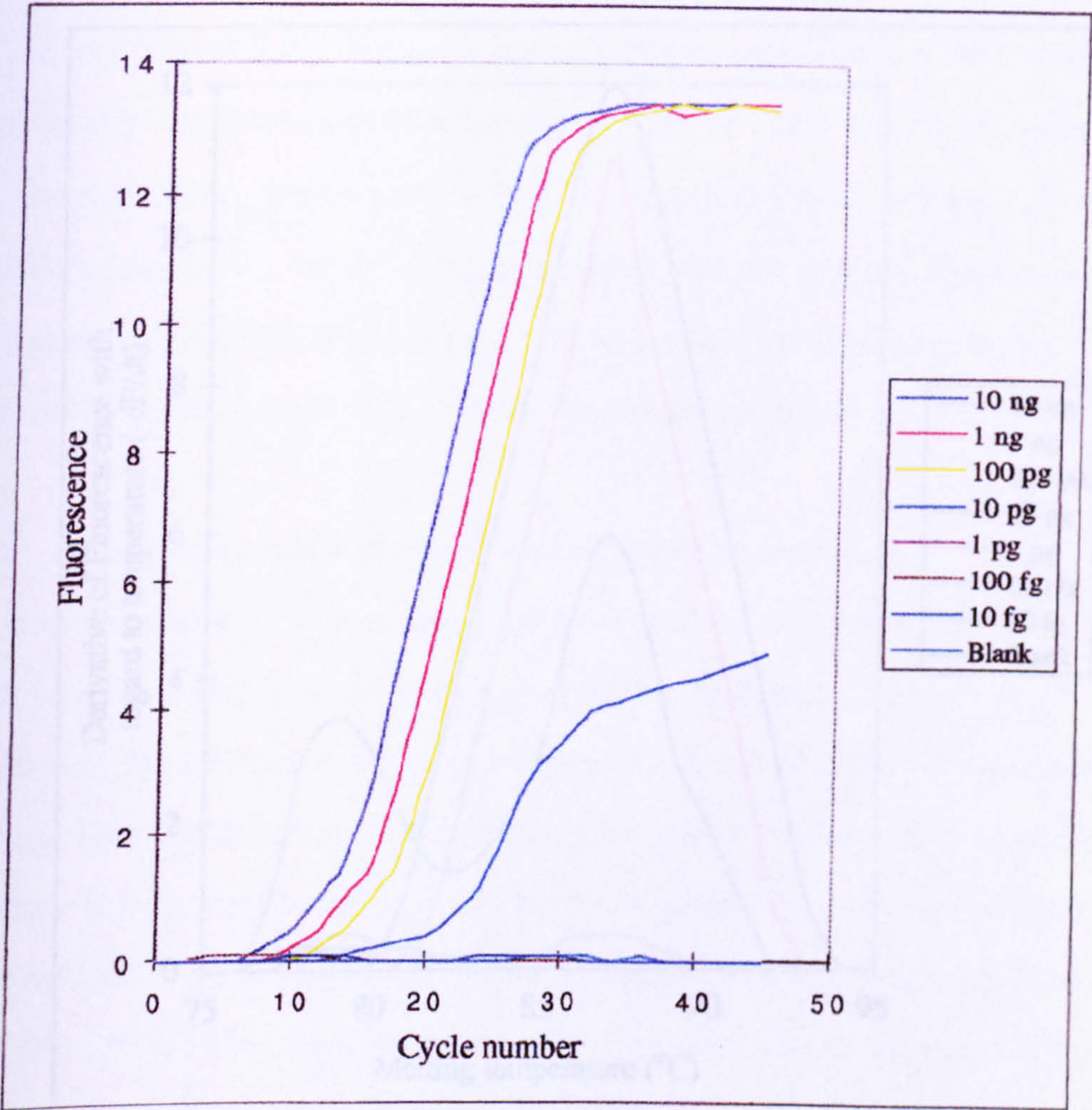


Figure 5.9a The effect of 10 fg of primer dimers on the amplification of a dilution series of a 900 bp target using *AmpliTaq Gold*[™] DNA polymerase. Samples were amplified for 45 cycles using the standard methodology described in Section 2.16.4, prior to melting curve analysis.

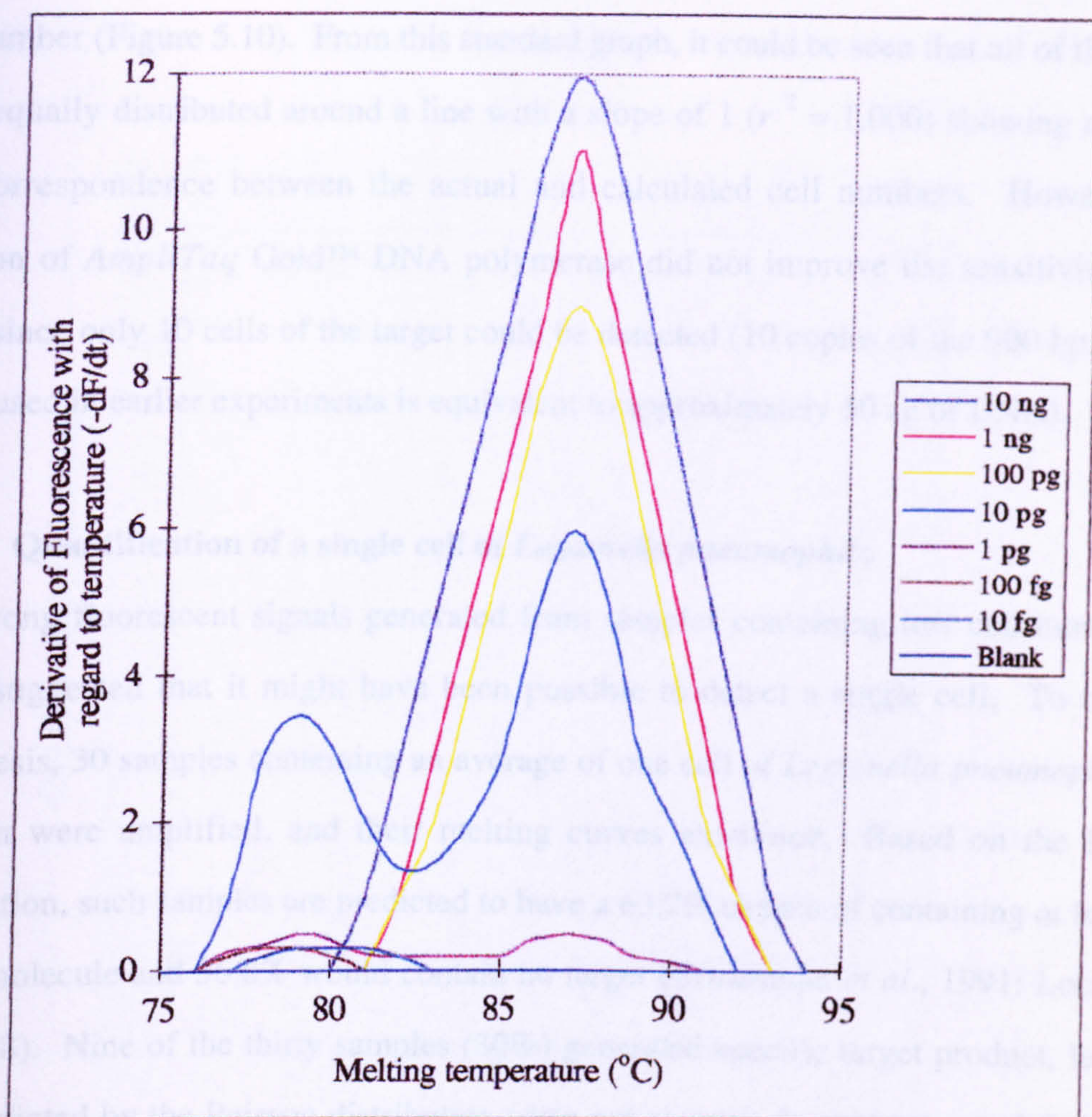


Figure 5.9b Melting curve analysis of a dilution series of a 900 bp target using *AmpliTaq Gold™* DNA polymerase in the presence of 10 fg of primer dimers. Melting curves were obtained after 30 seconds of annealing at 74°C, followed by a 0.1°C s⁻¹ linear temperature transition to 94°C.

Quantification of *Legionella pneumophila* cells over 5 orders of magnitude was performed using *AmpliTaq Gold*[™] DNA polymerase. A standard graph, consisting of 9 points, was constructed. The calculated cell number was plotted as a function of actual cell number (Figure 5.10). From this standard graph, it could be seen that all of the points were equally distributed around a line with a slope of 1 ($r^2 = 1.000$) showing a one-to-one correspondence between the actual and calculated cell numbers. However, the addition of *AmpliTaq Gold*[™] DNA polymerase did not improve the sensitivity of the assay since only 10 cells of the target could be detected (10 copies of the 900 bp plasmid target used in earlier experiments is equivalent to approximately 50 ag of DNA).

5.2.5 Quantification of a single cell of *Legionella pneumophila*

The strong fluorescent signals generated from samples containing low cell numbers of target suggested that it might have been possible to detect a single cell. To test this hypothesis, 30 samples containing an average of one cell of *Legionella pneumophila* per reaction were amplified, and their melting curves examined. Based on the Poisson distribution, such samples are predicted to have a 63.2% chance of containing at least one target molecule and 36.8% would contain no target (Brinchman *et al.*, 1991; Lockley *et al.*, 1998). Nine of the thirty samples (30%) generated specific target product, less than that predicted by the Poisson distribution (data not shown). In contrast, amplification of twenty reactions theoretically containing 10 cells of *Legionella pneumophila* generated positive signals in all instances (Figure 5.11).

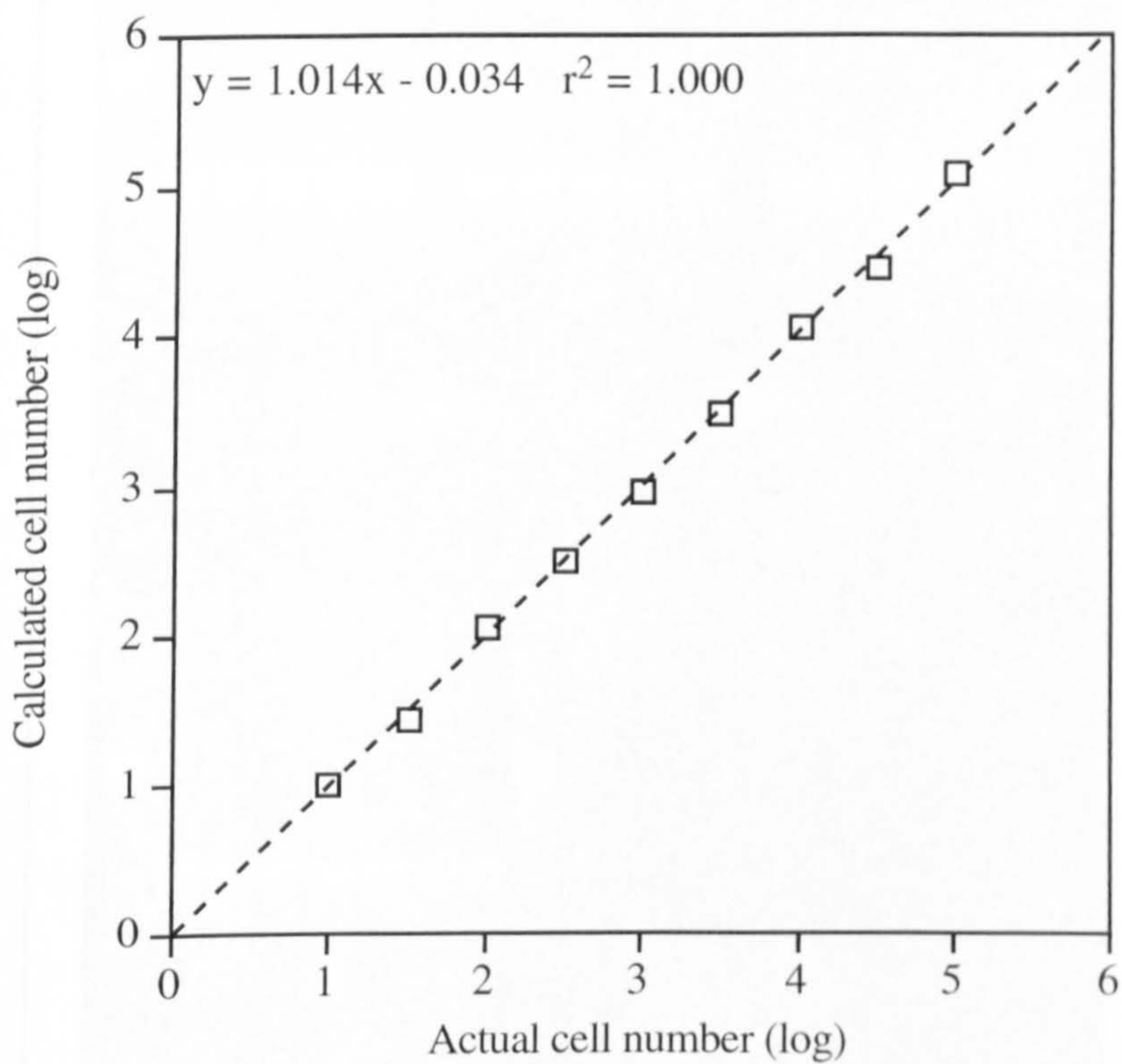


Figure 5.10 Standard graph for the quantification of *Legionella pneumophila* amplified using *AmpliTaq Gold™* DNA polymerase. The log of actual cell numbers was plotted against the log of calculated cell numbers. The dashed line represents a linear regression fit ($r^2 = 1.000$) to all the data points. Samples were amplified for 45 cycles using the standard methodology described in Section 2.16.4.

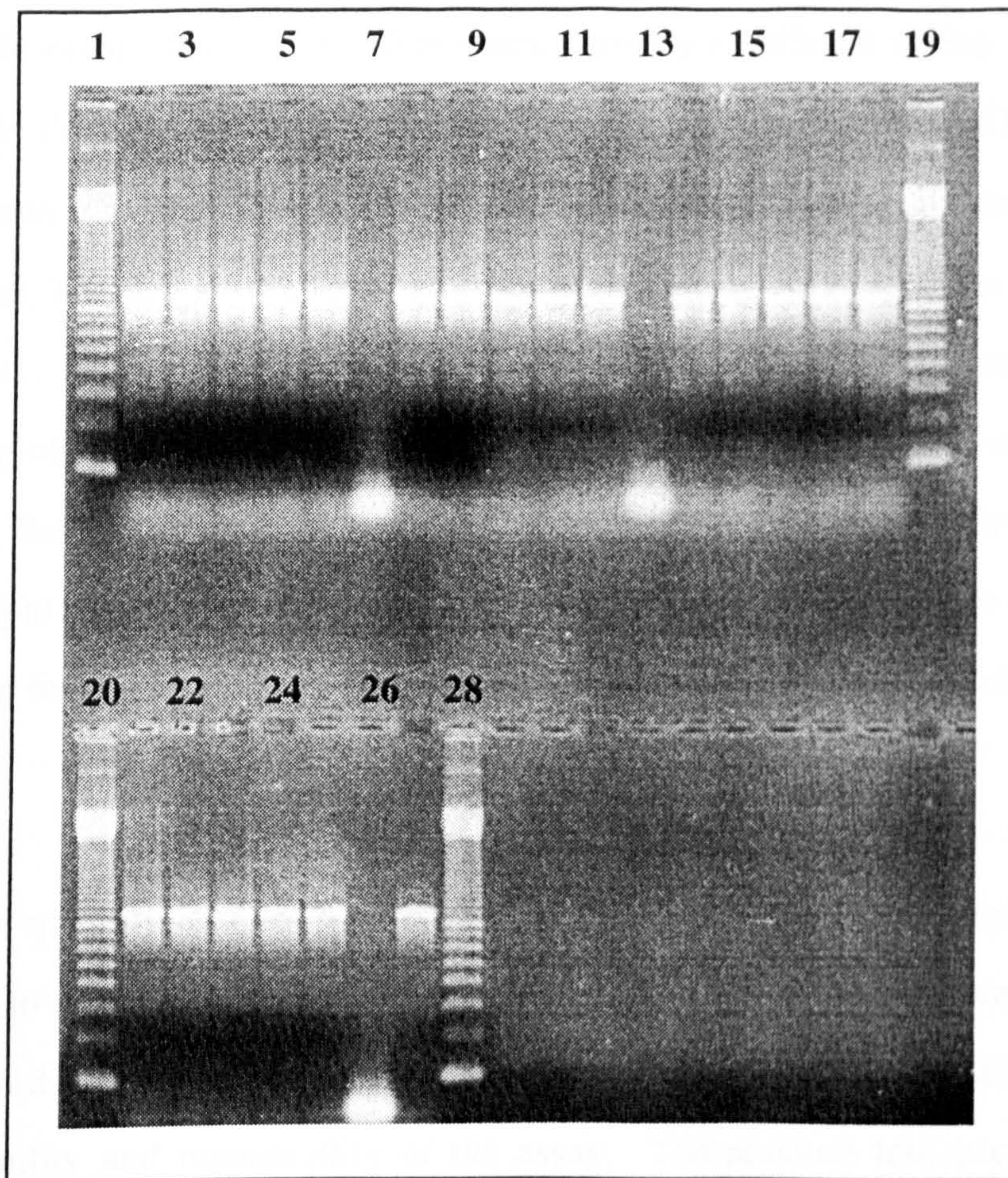


Figure 5.11 Agarose gel analysis of PCR products generated from reactions containing 10 cells of *Legionella pneumophila*. Lanes 1, 19, 20 and 28, 100 bp ladder; lanes 7, 13 and 26, negative controls; lanes 2-6, 8-12, 14-18 and 21-25 replicate reactions containing 10 cells of *Legionella pneumophila*; lane 27; 900 bp mimic (100 ng).

5.3 Discussion

Continuous fluorescence monitoring of DNA amplification simplifies the quantification of DNA because of its tremendous dynamic range (Higuchi *et al.*, 1993; Wittwer *et al.*, 1997a). Relatively new technologies such as the LightCycler enabled 45 temperature cycles to be completed in 10 to 20 minutes, allowing significant savings in analysis time to be made (Wittwer *et al.*, 1994; Wittwer *et al.*, 1997b). Using a dsDNA dye such as SYBR Green ITM (Higuchi *et al.*, 1993) not only allowed DNA synthesis to be monitored in real time, but also allowed post amplification melting curve analysis to be performed (Ririe *et al.*, 1997). The sensitivity of quantification with dsDNA dyes depends on the specificity of the amplification. As the specificity of amplification and detection are improved, the sensitivity of quantification with dsDNA dyes will also improve. For this work a rapid thermal cycler with fluorescence capability was used to monitor dsDNA production during each cycle by measuring the fluorescence of the dsDNA dye SYBR Green ITM.

In order to quantify low levels of a specific target such as *Legionella pneumophila* it is necessary to establish a highly sensitive PCR assay. Employing melting curves for the analysis and quantification of competitive PCR products is ultimately reliant on the reproducibility and repeatability of the assay. Temperature transition rates and dye concentration can affect the position and width of the melting curve obtained (Ririe *et al.*, 1997). Also, the addition of intercalators like ethidium bromide increases the melting temperature and broadens the temperature transition (Ririe *et al.*, 1997). However, the reproducibility and repeatability of the assay were not affected by these factors suggesting this type of assay could be used routinely with environmental samples.

Figure 5.7a shows a typical example of the fluorescence profile generated each cycle during the amplification of a serially diluted 900 bp template. As expected, the lower the initial starting concentration, the more cycles required for product detection (Wittwer *et al.*, 1997a). However, the fluorescence profiles generated using low initial starting

concentrations of the 900 bp template were non-existent, thereby limiting the sensitivity to starting concentrations greater than 1 pg (this is equivalent to 1.9×10^5 copies of the target or 231 cells of *Legionella* assuming one copy of the target per cell; Bej *et al.*, 1991). Also, the blank had an amplification profile similar to those samples containing less than 1 pg, indicating that at the detected signal was not produced by the specific product. Melting curve analysis of these (Figure 5.7b) and other samples identified non-specific amplification as the problem associated with using dsDNA dyes for quantification (data not shown). Typically, after 45 cycles, a PCR starting with 1 pg of target DNA ($\equiv 1.9 \times 10^5$ copies), consisted of two populations, specific products and non-specific products. To reduce the signal generated by non-specific products *AmpliTaq Gold*TM DNA polymerase was substituted for *Taq* DNA polymerase in reactions. This hot-start approach eliminated the formation of non-specific products (Figures 5.8a and 5.8b) allowing the quantification of 100 fg of target (this is equivalent to 1.9×10^4 copies of the target or 23 cells of *Legionella*). The addition of 10 fg of a synthetically constructed primer dimer to these reactions reversed the effect of *AmpliTaq Gold*TM DNA polymerase (Figures 5.9a and 5.9b) decreasing the overall amplification efficiency of the reaction (Table 5.5). This was consistent with the results obtained using *Taq* DNA polymerase, emphasising the effect of non-specific product formation on the accuracy of quantification.

Another method adopted to reduce non-specific signal took advantage of the tendency for specific and non-specific products to have differing T_m s. For example, the contribution of non-specific double-stranded products in the reactions using a 900 bp target was also eliminated by collecting fluorescence data at a temperature above the upper T_m limit of the non-specific products ($\approx 81^\circ\text{C}$). Although not observed in this work, there might be cases in which the non-specific products have a higher T_m than the specific products. In this instance, a subtractive method could be used in which fluorescence is acquired just above and just below the specific product T_m . In general, selective measurement of specific product could be used in any PCR in which the specific and non-specific

products have sufficiently different T_m s (e.g. their melting curves do not significantly overlap). The specific products generated by the Leg primers had higher T_m s than the non-specific products, and therefore fluorescence was acquired at 2°C below the specific product T_m .

When both methods were combined, quantification of 10 cells (\equiv 50 fg of genomic DNA) of *Legionella pneumophila* was possible (10 copies of the 900 target \equiv 50 ag of plasmid DNA). Plotting the initial cell number against the fractional cycle number (obtained at the fluorescence threshold) generated a linear slope. This relationship was used to construct a standard graph for quantification of unknown samples (Figure 5.10). The sensitivity of this protocol was similar to that obtained with more cumbersome fluorescence-based resonance energy transfer methods but did not require sequence-specific oligonucleotide probes (Lockley *et al.*, 1998). Quantification of known cell numbers of *Legionella pneumophila* showed that all of the points plotted were equally distributed around a line with a slope of 1 ($r^2 = 1.000$) showing a one-to-one correspondence between the actual and calculated cell numbers. These results suggest that this methodology should be applicable to any PCR based experiment.

The level of sensitivity obtained using the assay to quantify the 900 bp plasmid construct, compared to the actual *legionella pneumophila* target itself, was similar. 1.9×10^4 copies of the 900 bp target could be detected (the equivalent of 23 cells of *Legionella*). When the assay was applied directly to samples containing known cell numbers of *Legionella pneumophila*, as few as 10 cells were detected suggesting the quantification of *Legionella* to be accurate. Reassaying museum samples of the tap water samples produced the same results suggesting the assay was indeed accurate in the original determination of *Legionella* cell number (Tulley, pers. com.).

Although a single cell per reaction could be distinguished from the absence of template, the formation of non-specific products and stochastic effects increased the variance in

results obtained from samples containing less than ten cells, with only nine out of thirty samples (30%) generating a positive result. This is less than that predicted by Poisson distribution. In contrast, amplification of twenty reactions theoretically containing 10 cells of *Legionella pneumophila* (Figure 5.11) generated positive signals in all instances confirming that the original determination of *Legionella* cell number was correct (theoretically 98% of the reactions containing 10 cells should generate a positive signal according to Poisson distribution).

In summary, the continuous fluorescence monitoring of PCR products proved to be a powerful technique for quantification, routinely detecting ten cells of *Legionella pneumophila*. However, it was not possible to quantify cell numbers below this value due to the formation of non-specific products generated during amplification.

CHAPTER 6

DEVELOPMENT OF A MODEL SYSTEM TO INVESTIGATE MISPRIMING AND THE AMPLIFICATION OF NON-SPECIFIC PRODUCTS DURING PCR

6. DEVELOPMENT OF A MODEL SYSTEM TO INVESTIGATE MISPRIMING AND THE AMPLIFICATION OF NON-SPECIFIC PRODUCTS DURING PCR

6.1 Introduction

From the results obtained in Chapter 5, it appears that the greatest existing problem for the absolute quantification of nucleic acids using PCR is the mispriming and the subsequent amplification of non-specific PCR products. Mispriming of the DNA template in PCR leads to a competition for the DNA polymerase and primers between the specific and misprimed products, which can distort quantitative measurements. Commonly used approaches to reduce mispriming include optimisation of the concentrations of magnesium, DNA polymerase, primers, template and the number of PCR cycles for each particular PCR product (Innis and Gelfand, 1990; Saiki, 1989). However, even in a fully optimised system, such as that used in Chapter 5, other approaches are obviously required to reduce mispriming and hence improve assay sensitivity.

In order to develop a method capable of reducing mispriming to improve assay sensitivity, it was first necessary to develop a model system in which conditions analogous to those created during the amplification of low copy numbers of target DNA, were generated (e.g. the mispriming and amplification of non-specific products). This would allow the investigation of methods to prevent mispriming and the generation of non-specific products. To establish these conditions, a set of DNA standards, consisting of fragmented calf thymus DNA, were used in reactions and their affect on PCR amplification monitored in real time using the LightCyclerTM.

6.2 Results

6.2.1 The effect of degraded DNA on the amplification of target DNA

A set of DNA standards, consisting of fragmented calf thymus DNA, were degraded to varying extents by sonication for selected periods of time (Section 2.15). The resulting DNA after electrophoresis can be observed in Figure 6.1. Lane 3 contains the fragmented DNA prior to sonication containing a whole range of fragment sizes from the position of the well to approximately 100 bp. After only 5 seconds (lane 4) of sonication the size range of DNA fragments visible on the agarose gel had dropped to approximately 100-1200 bp, after 120 seconds (lanes 14) of sonication this size range was further reduced to approximately 100 bp to 500 bp.

In order to establish the effect of degraded DNA on the amplification of target DNA, a set of universal primers targeted against a conserved 16S rRNA gene sequence (U1 - CATGTATTAGGCTGCAATGC; U2; - CCGAATTACGATTTCGCATA), were used to amplify the sonication series of calf thymus DNA. The results show that two PCR products, approximately 300 bp and 600 bp in length, were generated using this set of primers (Figure 6.2). The smaller of the two amplimers was visible by gel analysis even when the most degraded calf thymus DNA was used as template (lane 15). However, the larger product was only intermittently generated (lanes 4-8 and lanes 11-13 respectively), with no 600 bp product visible in the most degraded DNA samples (lanes 14 and 15). Figure 6.2 also shows that increasing amounts of non-specific products ranging from 200-300 bp in size were generated with the amplification of increasingly fragmented template.

To investigate whether this inhibition was solely due to the loss of the 600 bp target site through fragmentation of the template, a series of samples consisting of approximately 5% (15 ng) high molecular weight calf thymus DNA and 95% (285 ng) degraded calf thymus DNA (sonicated for selected periods of time) were generated. The 15 ng of high molecular weight DNA alone was known to be sufficient for amplification (results not

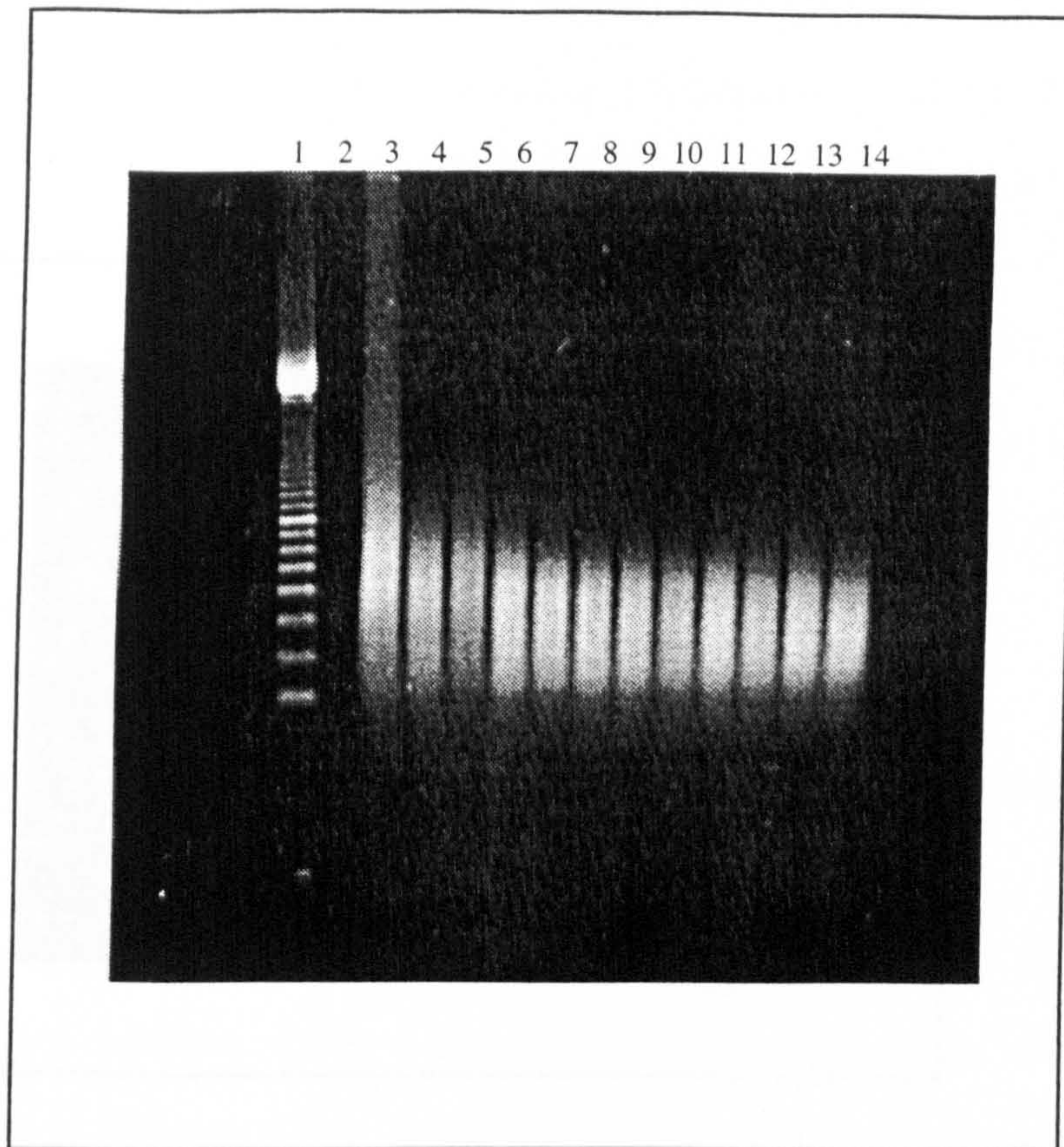


Figure 6.1 Agarose gel analysis of calf thymus DNA sonicated for varying lengths of time.

Figure 6.1 Agarose gel analysis of calf thymus DNA sonicated for varying lengths of time. Lane 1, 100 bp ladder; lane 2, negative control; lane 3, 0 seconds; lane 4, 5 seconds; lane 5, 10 seconds; lane 6, 20 seconds; lane 7, 30 seconds; lane 8, 40 seconds; lane 9, 50 seconds; lane 10, 60 seconds; lane 11, 70 seconds; lane 12, 80 seconds, lane 13, 90 seconds; lane 14, 120 seconds.

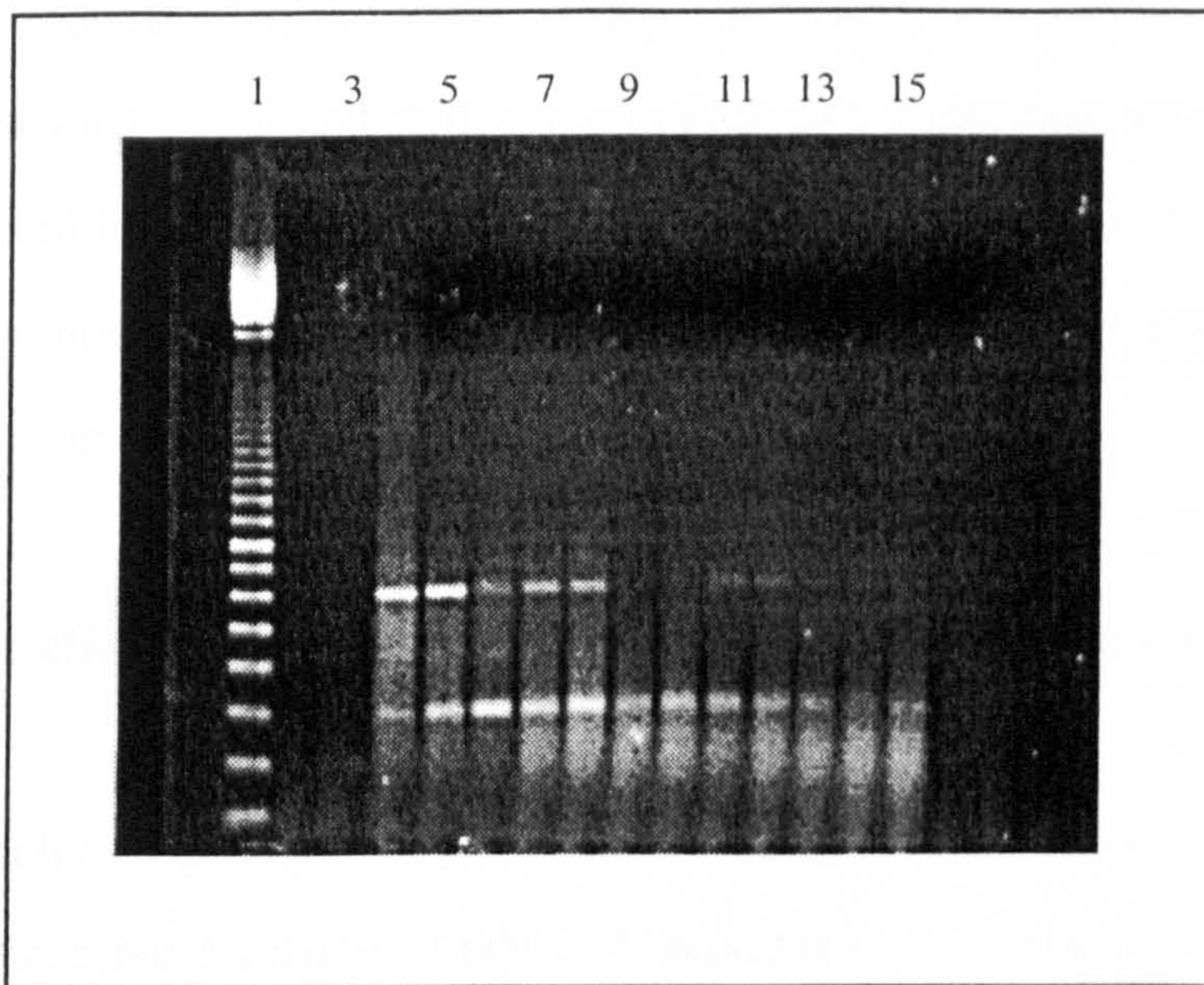


Figure 6.2 PCR products generated following amplification of sonicated calf thymus DNA. Lane 1, 100 bp ladder; lane 2, negative control; lane 3, blank; lane 4, 0 seconds; lane 5, 5 seconds; lane 6, 10 seconds; lane 7, 20 seconds; lane 8, 30 seconds; lane 9, 40 seconds; lane 10, 50 seconds; lane 11, 60 seconds; lane 12, 70 seconds; lane 13, 80 seconds; lane 14, 90 seconds, lane 15, 120 seconds.

shown). These mixed samples, all of which should therefore contain sufficient intact target, were amplified using the universal primers (Figure 6.3). A reduction in the yield of both the 600 and 300 bp product, seen as a decrease in the intensities of the bands on the agarose gel, was associated with increased degradation of the template. Once again, increasing amounts of non-specific products ranging in size from 200-300 bp in size were generated with the amplification of an increasingly fragmented template. The shorter 300 bp product was consistently amplified in all of the sonicated DNA samples (lanes 4-10) although a reduction in the yield of degraded DNA was observed. The larger PCR product was only generated with the template mix containing degraded DNA that had been sonicated for 70 seconds or less (lanes 4-9). It appears therefore that the presence of increasingly more degraded DNA was progressively more inhibitory to amplification due to the formation of non-specific products.

6.2.2 The effect of 3'-end labelling degraded DNA on the amplification of target template

Haptens, such as digoxigenin (DIG), have been used routinely to 3'-end label probes for the non-radioactive detection of DNA. It was therefore decided to use this moiety to 3'-end label degraded DNA to see if mispriming, caused by the increased presence of degraded target template, could be prevented and thus allow the amplification of the larger 600 bp target.

In order to test this hypothesis, a sonication series of calf thymus DNA possessing 3'-ends labelled with ddUTP digoxigenin (DIG) (Section 2.15.1), was supplemented in place of that used in the experiment shown in Figure 6.3, i.e. mixed in the same proportions with high molecular weight genomic DNA using the same set of primers and PCR conditions for amplification. Amplification of this sonication series is shown in Figure 6.4. The 600 bp product was generated in the most degraded DNA samples (lanes 8 and 9) confirming that hybridised DNA fragments were acting as substrates for extension by *Taq* DNA polymerase.

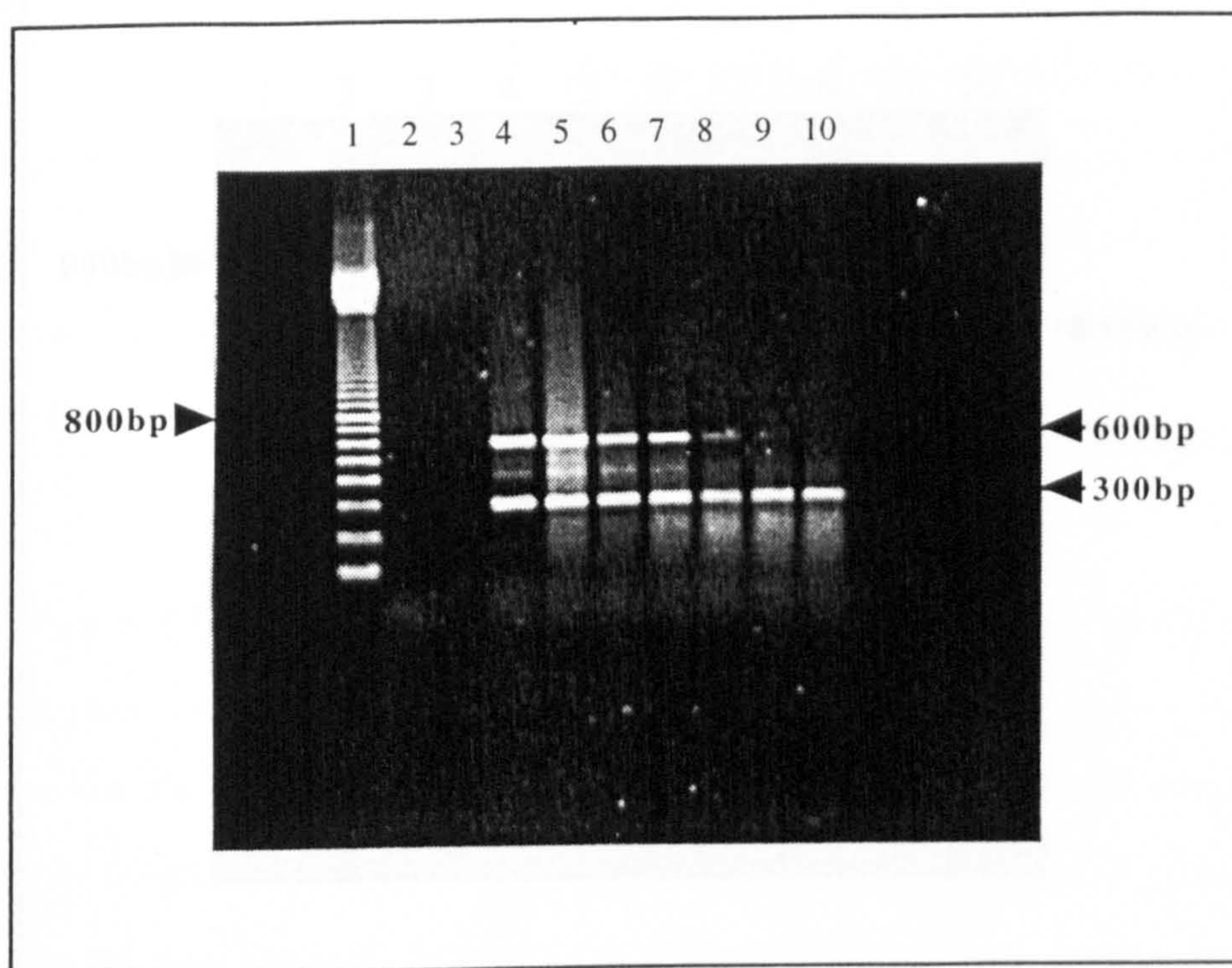


Figure 6.3 The effect of degraded DNA on PCR using universal primers. Lane 1, 100 bp ladder; lane 2, negative control; lane 3, blank; lane 4, HMW calf thymus DNA; lanes 5 to 10, admixtures of HMW and degraded calf thymus DNA (70% sonicated + 30% normal calf thymus DNA); lane 5, 5 seconds; lane 6, 10 seconds; lane 7, 30 seconds; lane 8, 50 seconds; lane 9, 70 seconds; lane 10, 90 seconds.

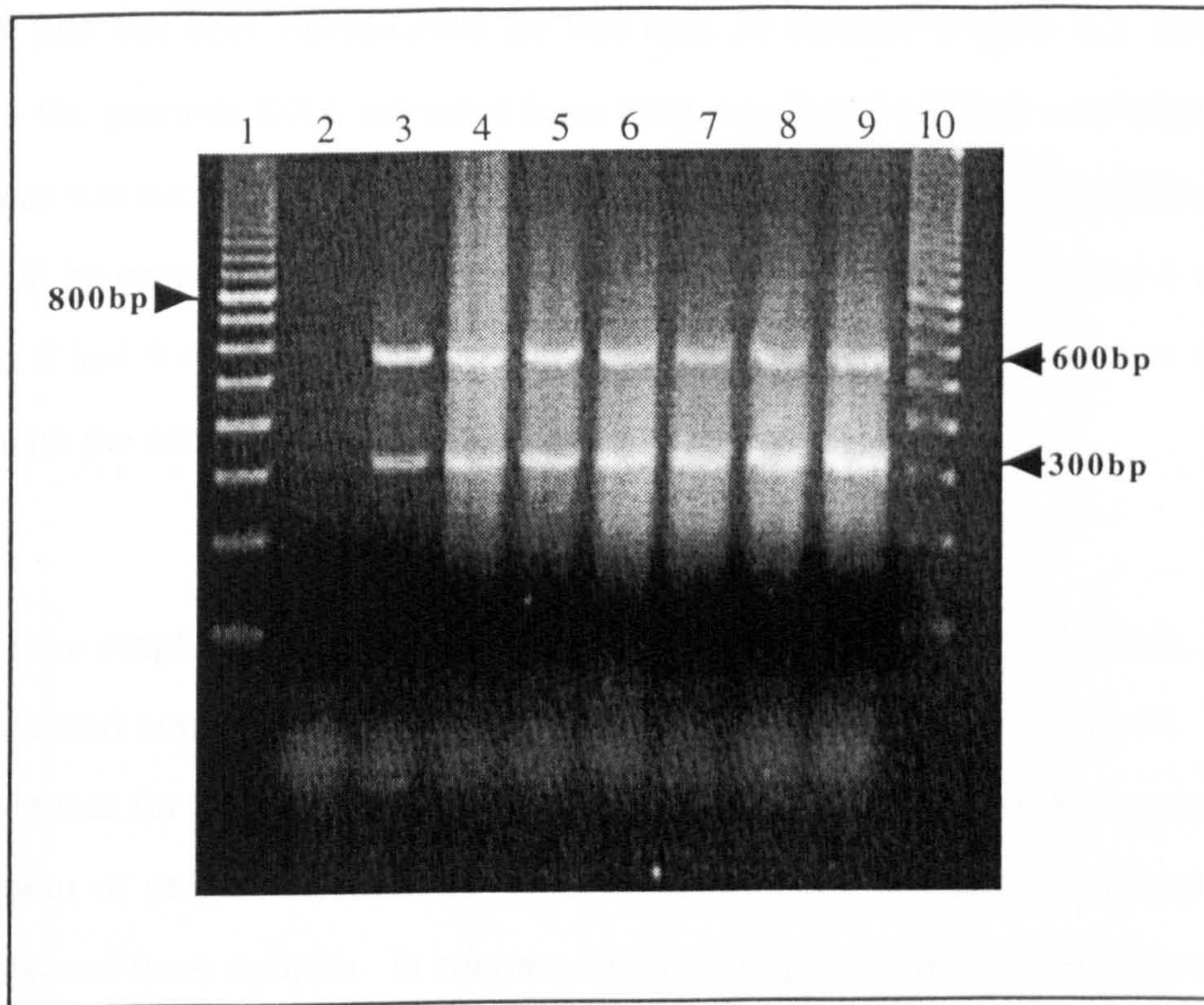


Figure 6.4 The effect of 3'-end labelling degraded DNA prior to PCR. Lane 1, 100 bp ladder; lane 2, negative control; lane 3, HMW calf thymus DNA; lanes 4 to 9, admixtures of HMW and degraded Calf thymus DNA (70% sonicated + 30% normal calf thymus DNA); lane 4, 0 seconds; lane 5, 10 seconds; lane 6, 30 seconds; lane 7, 50 seconds; lane 8, 70 seconds; lane 9, 90 seconds; lane 10, 100 bp ladder.

To apply this end labelling technique to a more realistic sample, in which conditions analogous to those created during the amplification of low copy numbers of target DNA, were generated (e.g. the mispriming and amplification of non-specific products), beef muscle tissue was microwaved for various lengths of time and the DNA extracted (Section 2.15.1). The longer 600 bp product was only amplified in reactions containing DNA that had been microwaved for less than 30 seconds (Figure 6.5, lanes 3 and 4). When the genomic DNA extracted from meat was 3'-end labelled with DIG, the 600 bp product was successfully generated in all of the reactions (Figure 6.6) although yields of the 600 bp product were slightly reduced from samples microwaved for 4 to 5 minutes (lanes 8 and 9 respectively). No non-specific products were observed on either of the gels with the meat amplified products.

Real time amplification and PCR product melting curve analysis (Section 2.16.4) was then carried out on all microwaved meat samples using the Lightcycler™. Figure 6.7a showed that for the amplification of the non-labelled template, the PCR reaction reached a plateau of amplification at the same level for the non-microwaved and 30 second microwaved meat samples. In contrast, the level of plateau representing the total amount of product generated was reduced with increased microwaving and degradation of the template. This was presumably due to the loss of the 600 bp product as seen in Figure 6.5. Figure 6.7b shows the melting curve analysis of PCR products generated from the non-labelled DNA. The 600 bp product is shown to have a melting temperature of approximately 87°C whilst non-specific products a T_m of approximately 78-81°C. However, these non-specific products were equivalent to those produced in the "no DNA" control reaction and may therefore be due primarily to the formation of primer dimers. The same analysis using the Lightcycler™ was carried out on the 3'-DIG-labelled meat DNA. The amplification profiles (Figure 6.8a) revealed that the meat microwaved for 0-

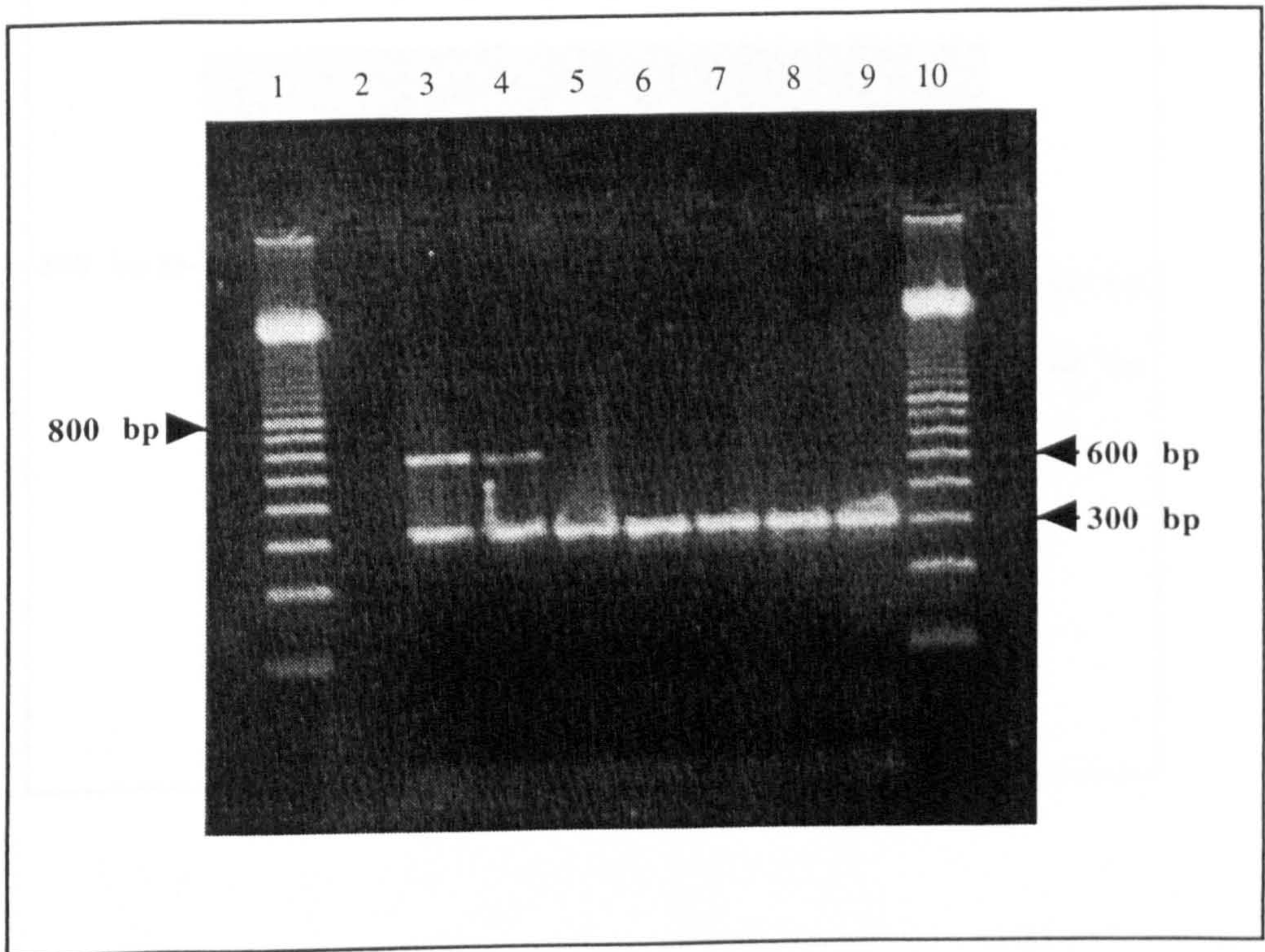


Figure 6.5 The effect of degrading DNA on the PCR of beef samples using universal primers. Lane 1, 100 bp ladder; lane 2, negative control; lanes 3 to 9, microwaved beef samples; lane 3, 0 seconds; lane 4, 30 seconds; lane 5, 1 minute; lane 6, 2 minutes; lane 7, 3 minutes; lane 8, 4 minutes; lane 9, 5 minutes; lane 10, 100 bp ladder.

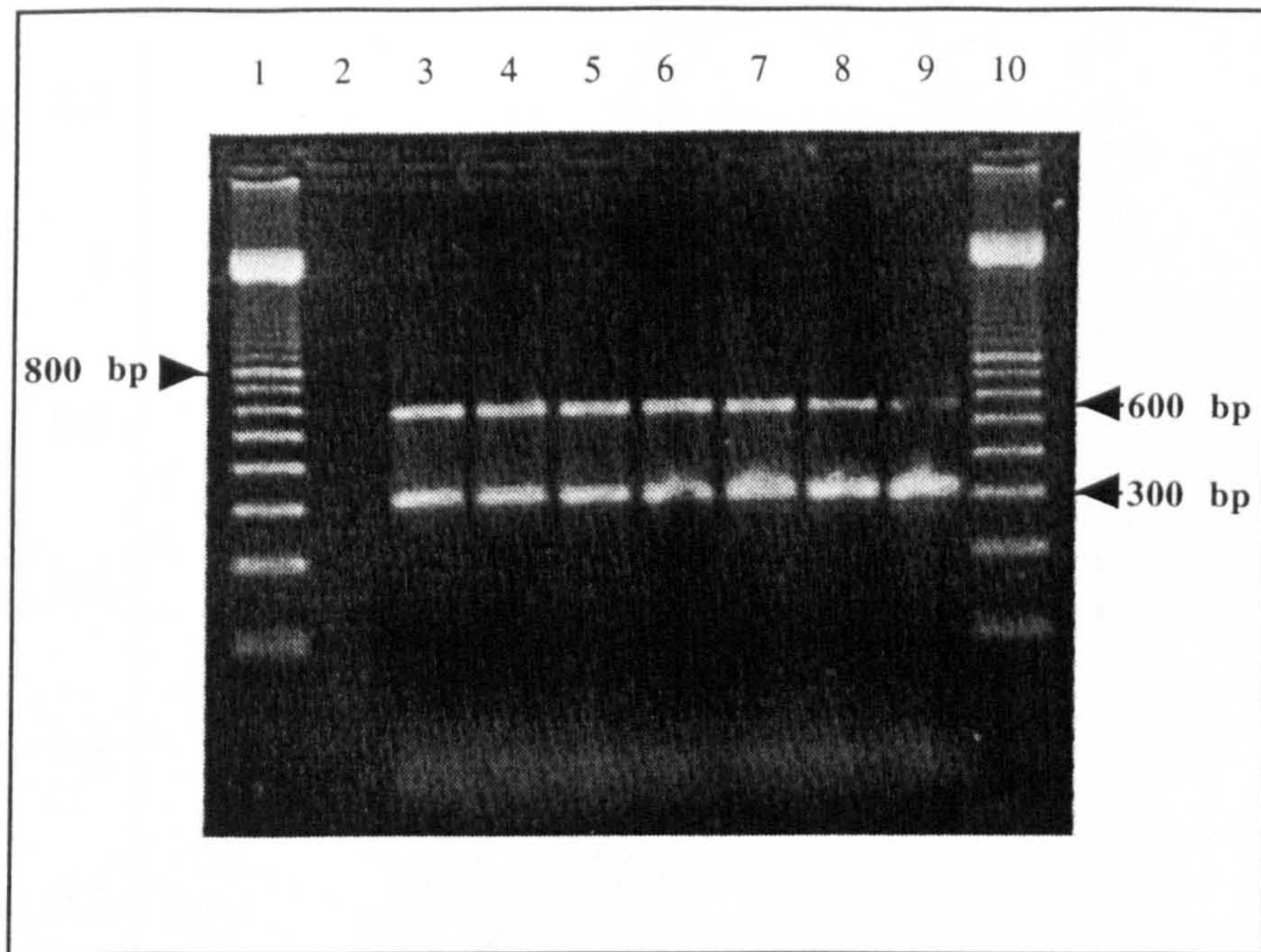


Figure 6.6 The effect of 3'-end labelling degraded DNA obtained from microwaved beef samples prior to PCR. Lane 1, 100 bp ladder; lane 2, negative control; lanes 3 to 9, microwaved beef samples; lane 3, 0 seconds; lane 4, 30 seconds; lane 5, 1 minute; lane 6, 2 minutes; lane 7, 3 minutes; lane 8, 4 minutes; lane 9, 5 minutes; lane 10, 100 bp ladder.

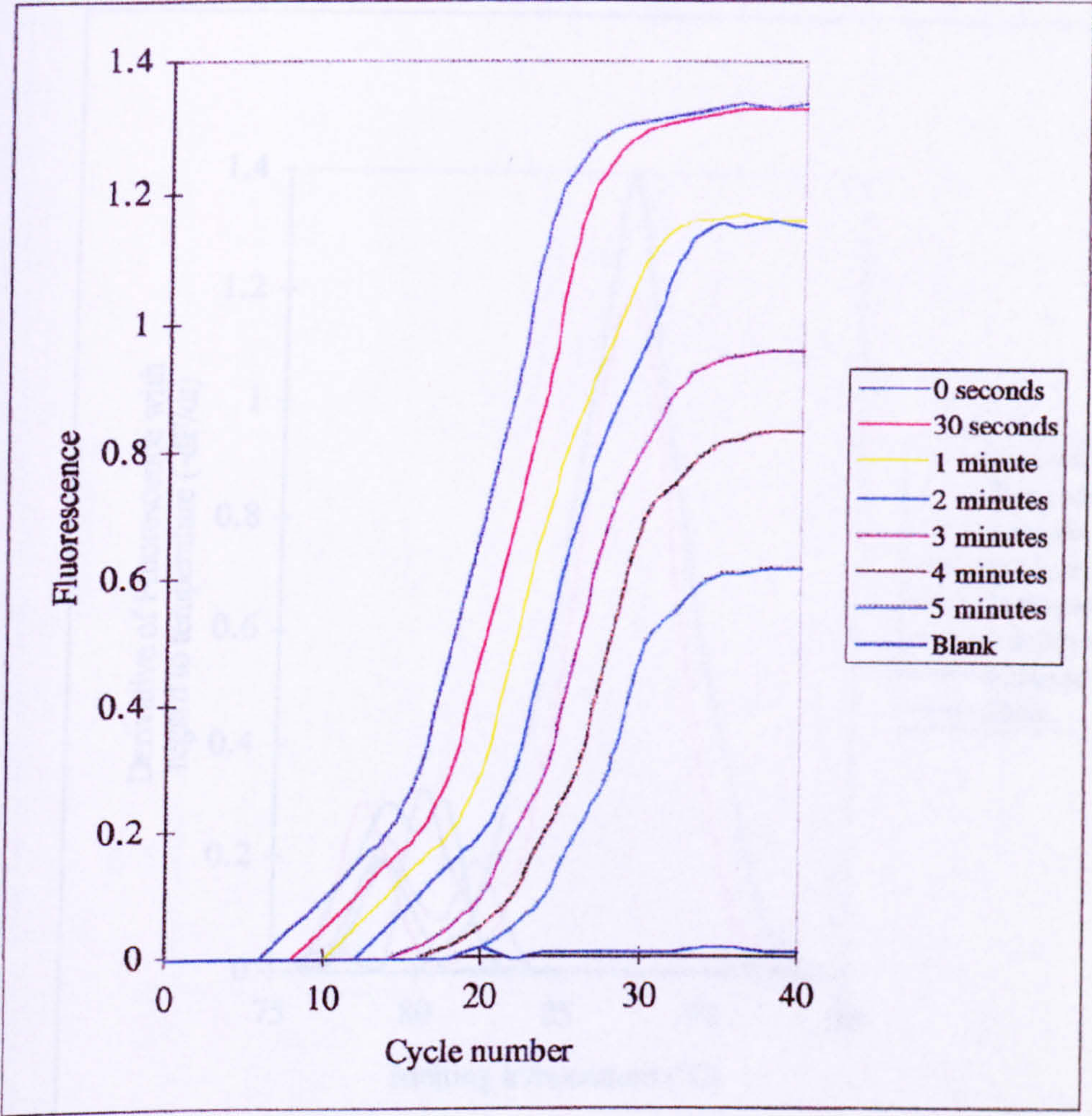


Figure 6.7a Fluorescence profiles of a 600 bp target generated from the amplification of degraded DNA obtained from microwaved beef samples using universal primers. Samples were amplified for 45 cycles using the thermal cycling conditions described in Section 2.16.4, prior to melting curve analysis.

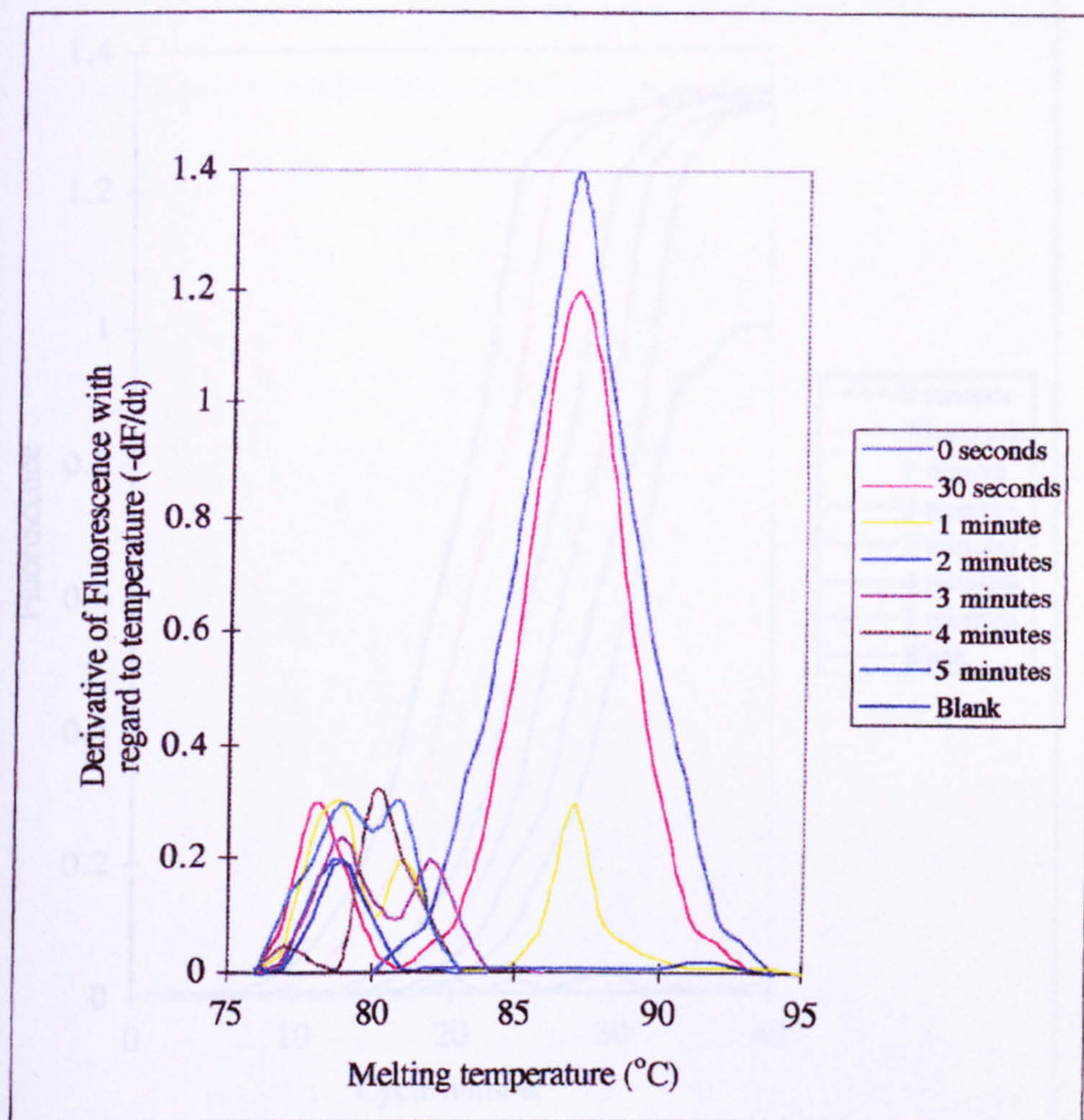


Figure 6.7b Melting curve analysis of 600 bp products generated from the amplification of degraded DNA obtained from microwaved beef samples using universal primers. Melting peaks obtained following amplification confirmed the heterogeneity of the products generated. Samples microwaved for more than 30 seconds generated peak profiles indicative of non-specific amplification products. Melting curves were obtained after 30 seconds of annealing at 74 $^{\circ}\text{C}$, followed by a 0.1 $^{\circ}\text{C s}^{-1}$ linear temperature transition to 94 $^{\circ}\text{C}$.

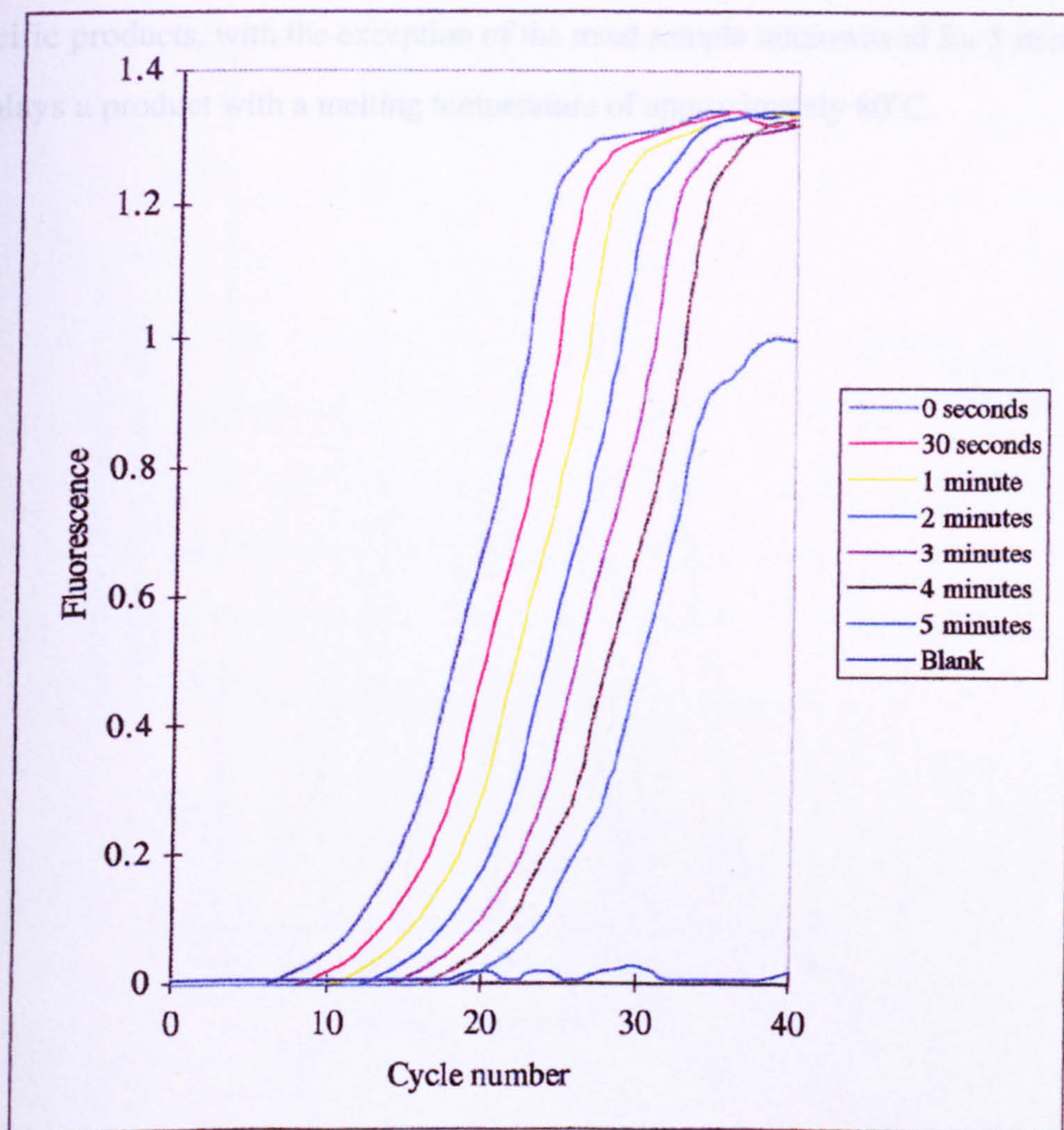


Figure 6.8a Fluorescence profiles of 600 bp products generated from the amplification of degraded DNA obtained from microwaved beef samples 3'-end labelled with DIG. Samples were amplified for 45 cycles using the thermal cycling conditions described in Section 2.16.4, prior to melting curve analysis.

4 minutes prior to end labelling all reached plateau at the same level with only the sample microwaved for 5 minutes having a reduced plateau level. Melting curve analysis of these samples (Figure 6.8b) showed a significant reduction in the generation of non-specific products, with the exception of the meat sample microwaved for 5 minutes which displays a product with a melting temperature of approximately 80°C.

6.3 Discussion

At 94°C, according to thermodynamic calculations, all of the amplified products should be completely dissociated. Under normal PCR conditions, products are present in vast excess, therefore in the early PCR cycles most of the degraded products should become bound to a primer when the temperature drops in the annealing step. However, if a large population of denatured and fragmented template DNA is also present, these may also anneal either to the high molecular weight template DNA or to each other, extension of these self-annealed fragments could then proceed with the same efficiency as events for Tag polymerase and dNTPs. This would lead to the formation of non-specific products. The linear phase of the amplification curve is a single step and may or may not contain reaction components. However, the reaction mixture will only be as efficient in the later cycles as the initial reaction mixture. The stages of PCR as examined by the Linear Amplification Curve (LAC) method.

Although the 3'-end DIG labelling of the template DNA was not designed for double-stranded template, it is possible that some of the template DNA was double-stranded and was present in the degraded template. The efficiency of end-labelling was not examined, however, but the results suggest that a sufficient amount of end-labelling had occurred to affect the amplification of the degraded template.

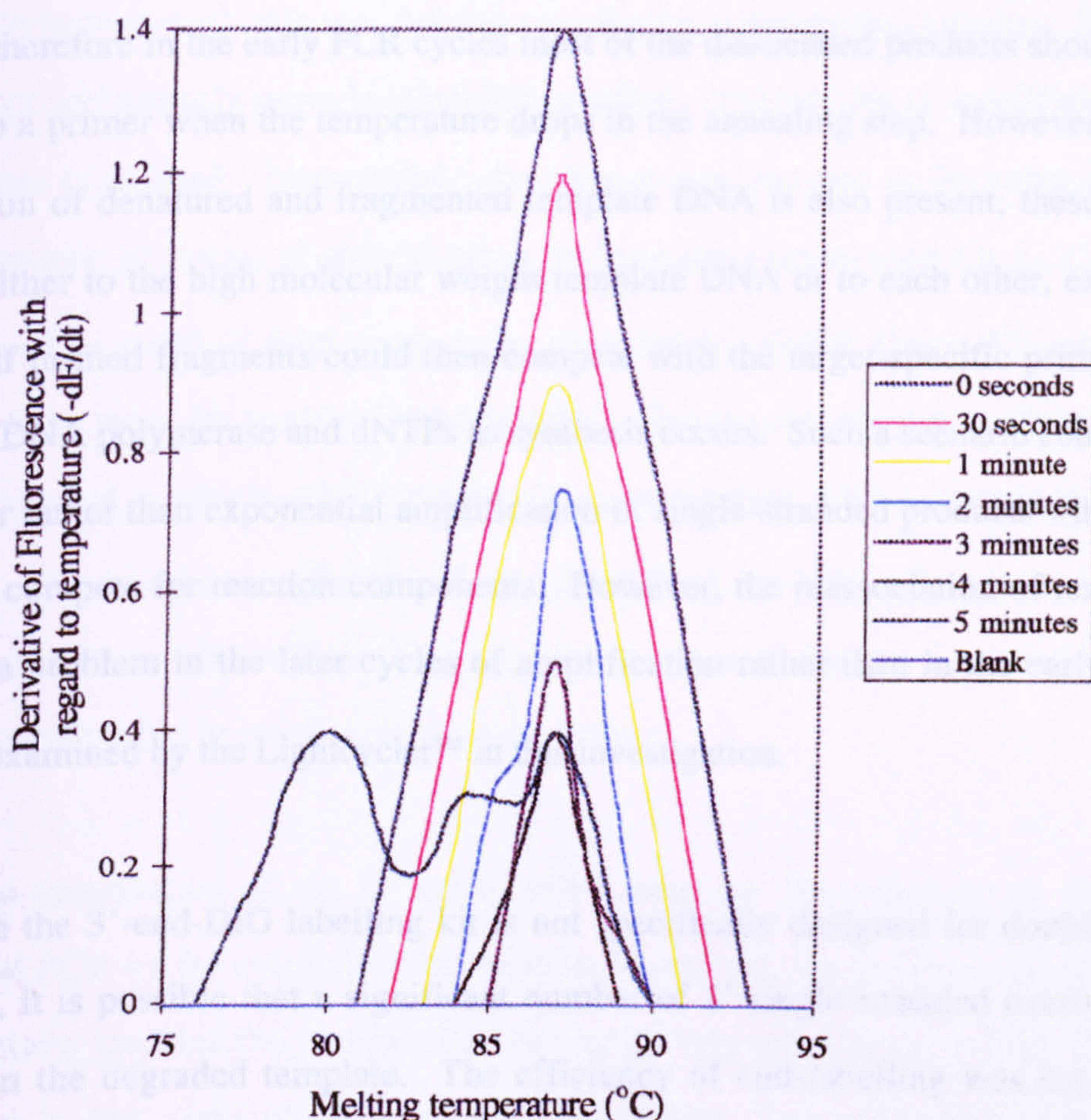


Figure 6.8b Melting curve analysis of 600 bp products generated from the amplification of degraded DNA obtained from microwaved beef samples 3'-end labelled with DIG. Analysis of the melting peaks confirmed the purity of the products. Melting curves were obtained after 30 seconds of annealing at 74°C, followed by a 0.1°C s⁻¹ linear temperature transition to 94°C.

6.3 Discussion

At 94°C, according to thermodynamic calculations, all of the amplicon products should be completely dissociated. Under normal PCR conditions, primers are present in vast excess, therefore in the early PCR cycles most of the dissociated products should become bound to a primer when the temperature drops in the annealing step. However, if a large population of denatured and fragmented template DNA is also present, these may also anneal either to the high molecular weight template DNA or to each other, extension of these self primed fragments could then compete with the target-specific priming events for *Taq* DNA polymerase and dNTPs as synthesis occurs. Such a scenario could result in the linear rather than exponential amplification of single-stranded products which may or may not compete for reaction components. However, the reassociation of template will only be a problem in the later cycles of amplification rather than in the early stages of PCR as examined by the Lightcycler™ in this investigation.

Although the 3'-end-DIG labelling kit is not specifically designed for double-stranded template, it is possible that a significant number of 3' single-stranded overhangs were present in the degraded template. The efficiency of end-labelling was not examined however, but the results suggest that a sufficient amount of end-labelling had occurred to affect the amplification of the degraded template.

The shift in the linear phase of the amplification profile representing the total yield of PCR products (fluorescence plotted against cycle number) was similar to that observed when reduced copy numbers of template are added to PCR reactions (described in Chapter 5). In this instance the shift effect seen in Figures 6.7a and 6.8a could therefore be attributed to the reduction in intact targets due to the degradation of the template by microwaving. The linear phase of the amplification profile for both the non-labelled and 3'-end labelled meat DNA was of a similar slope suggesting that during the cycles when this slope was attained their amplification efficiencies were approximately similar.

However, whereas amplification at this level of efficiency was maintained over the same number of cycles for the majority of the labelled samples (Figure 6.8a, 0-4 minutes microwaving), the exception being the most heavily degraded sample (Figure 6.8a, 5 minutes microwaving), this was not the case for the non-labelled samples. Figure 6.7a shows that increasingly degraded template results in fewer cycles of amplification before the plateau is reached. Interestingly, the plateau was attained at the same point, after approximately 30 cycles of amplification, for samples microwaved for 1, 2, 3, 4 and 5 minutes. The yield of total product (as detected by SYBR Green ITM intercalation) decreased the more the template was degraded. This is probably due to the formation of single-stranded products which may be more prevalent with increasingly degraded template. However, due to the specificity of SYBR Green ITM for double-stranded DNA, single-stranded products will not be detected and therefore will not be represented by the amplification profile or melting curve analysis data.

Increased degradation of the 3'-end labelled template DNA appears to increase the number of PCR cycles required before plateau is attained (Figure 6.8a), however, this is only partially true for the non-labelled degraded template (Figure 6.7a). Therefore a reduction in the yield of PCR products amplified from degraded DNA may be experienced if the reaction is not allowed to continue to the plateau phase. Increased degradation of the template DNA appears to increase the number of PCR cycles required before plateau is reached suggesting that template DNA fragments were hybridising and acting as substrates for extension. This suggests that disruption of the target site is not the sole cause of inhibition of the 600 bp product.

Sonication of calf thymus DNA generated fragments with an average size of approximately 200 bp (Figure 6.1 shows that there are approximately 3 times as many 100 bp fragments as there are 300 bp fragments). This means that there is an almost infinite variety of 100 bp-300 bp fragments at approximately 25% the concentration of the specific primers. A substantial amount of the bovine genome is single copy and will

not reanneal during the lifetime of the experiment (Roberts, pers. com.). The remaining part of the genome will consist of moderately-to-highly repetitive sequences (Hunt *et al.*, 1997) and will reanneal rapidly, probably within a single PCR cycle. Any free ends which lie in a repetitive region will anneal easily and direct extension by *Taq* DNA polymerase. Although the effective concentration of the short (100 bp-300 bp) DNA fragments is lower than that of the specific primer, the effective target concentration might be much higher (even than the repetitive rRNA target; some repetitive families have a copy number of 10^4 - 10^5 per genome) (Roberts, pers. com.). Having primed and been extended, many products will be more kinetically burdened and so will not reanneal during the experiment, accumulating as ssDNA. This is probably the reason for the lowering of the plateau phase observed in this system which primarily measures double-stranded DNA.

From the results obtained previously in Chapter 5, mispriming and the subsequent amplification of non-specific PCR products proved to be a problem for the absolute quantification of DNA using PCR. In this chapter we have shown that the mispriming of very short degraded DNA fragments can affect the amplification of a target. In conditions analogous to those created in this system, for instance during the amplification of low copy numbers of target DNA, this would lead to the generation of non-specific products preventing the absolute quantification of a target template (as discussed in Section 5.3). However, the 3'-end labelling of degraded calf thymus DNA and microwaved beef DNA with DIG prevented the extension of the short degraded DNA fragments showing that this strategy could be used successfully to prevent mispriming. It was therefore decided to use this approach to generate 5'-degenerate, 3'-DIG-terminated competitive primer analogues (discussed in Chapter 7), to try and block non-specific primer binding sites allowing the quantification of a single cell of *legionella pneumophila*.

CHAPTER 7

THE USE OF 5'-DEGENERATE, 3'- TERMINATED COMPETITIVE PRIMER ANALOGUES TO IMPROVE THE SPECIFICITY OF AMPLIFICATION USING THE LIGHTCYCLER™

7. THE USE OF 5'-DEGENERATE, 3'-TERMINATED COMPETITIVE PRIMER ANALOGUES TO IMPROVE THE SPECIFICITY OF AMPLIFICATION USING THE LIGHTCYCLER™

7.1 Introduction

Although a number of approaches are commonly used to prevent mispriming during PCR (discussed in Section 6.1) more recent studies have emphasised the importance of annealing temperature, competitive primer analogues and the use of modified DNA polymerases to improve the specificity of amplification (Hecker and Roux, 1996; Moretti *et al.*, 1998; Puskas and Bottka, 1995; Zhu and Clark, 1996). The use of tRNA to reduce the formation of non-specific products during PCR has also been employed to improve assay specificity (Stürzenbaum, 1999).

Puskas and Bottka (1995) demonstrated that the specificity of PCR amplification could be improved by using a 3'-terminated primer which would not be amplified by *Taq* DNA polymerase but, could anneal to sites of non-specific template-primer interaction, effectively blocking these sites. However, retarded amplification of specific products was observed when two 3'-terminated primers were used in a reaction. No attempt was made to do quantitative PCR.

Extending this idea further, Atamas *et al.*, (1998) used a set of 5'-degenerate, 3'-dideoxy-terminated competitive primer analogues to improve the specificity of amplification. These 5'-degenerate, 3'-terminated primer analogues were more competitive for less homologous sites that possessed some degree of homology to that of the original functional primer. This improved specificity because in a set of 5'-degenerate, 3'-

terminated primer analogues, most sequences will possess structural homology with the templates that are less specific for the functional primer but resemble the specific template (Zhu and Clark, 1996). Consequently, 5'-degenerate, 3'-dideoxy-terminated competitive primer analogues presumably anneal better to templates that are less specific for the functional primer and thus prevent the amplification of less specific products by blocking these sites (Hecker and Roux, 1996).

Due to the very high cost of synthesising 5'-degenerate, 3'-dideoxy-terminated competitive primer analogues another method was required to 3'-end label primers to prevent amplification by *Taq* DNA polymerase. Since the 3'-end labelling of degraded calf thymus and beef DNA with DIG prevented the extension of fragments by *Taq* DNA polymerase it was decided to use this approach to produce 5'-degenerate, 3'-DIG-terminated competitive primer analogues.

The aim of this investigation was therefore to design and use 5'-degenerate, 3'-DIG-terminated competitive primer analogues to prevent mispriming and consequently improve the specificity of PCR without affecting the quantification of the final specific product using the LightCycler™.

7.2 Results

7.2.1 Designing 5'-degenerate, 3'-DIG-terminated competitive primer analogues

There are a number of ways to design this type of primer analogue. The primer analogue could be an exact copy of the functional primer, a mixture of fully randomised oligonucleotides or a partially random primer resembling the functional primer but not copying it. The first type of primer analogue would inhibit specific and non-specific amplification whilst the fully random primers would anneal to a wide variety of irrelevant templates. These types of primers were therefore not considered suitable for the purpose of this investigation. However, partially random or “degenerate” primer analogues would be more competitive for sites that are less homologous, but still retain some degree of homology to the functional primer, thus increasing the specificity of amplification. Consequently, these degenerate primer analogues were selected for use to prevent mispriming and the subsequent amplification of non-specific products.

5'-degenerate, 3'-DIG labelled competitor analogues were, for the normal strand primer, 5'-NNN NNN NNN NNN CTC GCT G-DIG-3' and for the complementary primer, 5'-NNN NNN NNN NNN AGC TTC A-DIG-3'. In the analogue sequences, the N stands for random occurrence of any A, G, T or C base. The competitive primer analogues were used at a concentration of 3 µM in the PCR.

7.2.2 The effect of 5'-degenerate, 3'-DIG-terminated competitive primer analogues on amplification efficiency

Since 5'-degenerate, 3'-DIG-terminated competitive primer analogues were to be used in reactions it was first necessary to determine their effect on amplification efficiency. For this, selected cell numbers of *Legionella pneumophila* were amplified in the presence and absence of these analogues. The amplification efficiencies obtained in the presence of these analogues were all greater than those obtained in their absence (Table 7.1).

Table 7.1 The effect of 5'-degenerate, 3'-DIG-terminated competitive primer analogues on the amplification efficiencies of selected cell numbers of *Legionella pneumophila*.

Initial cell number	Amplification efficiency (E) (Analogues present)	Amplification efficiency (E) (Analogues absent)
10	91%	73%
10 ²	92%	79%
10 ³	94%	82%
10 ⁴	94%	83%

7.2.3 The effect of competitive primer analogues on assay specificity

The quantification of *Legionella pneumophila* was repeated in the presence of these competitive primer analogues. A standard graph was constructed similar to the one shown in Figure 5.6. Figure 7.1 shows a typical standard graph obtained for reactions containing initial cell numbers ranging from 10⁻¹ to 10⁵ cells per reaction. From the standard graphs generated it was possible to reliably detect 1 cell of *Legionella pneumophila* (this analysis was performed on at least twenty separate occasions). Melting curve analysis confirmed that the inclusion of the competitive primer analogues in reactions blocked the amplification of non-specific products increasing the sensitivity of the assay by an order of magnitude (Figure 7.2).

To confirm these results, 20 samples containing a calculated average of one cell of *Legionella pneumophila* per reaction were amplified in the absence and presence of the competitive primer analogues using the LightCycler™. Following amplification, 10 µl of the samples were loaded onto an agarose gel for analysis. Only four of the twenty samples (25%) amplified in the absence of competitive primer analogues generated specific products (data not shown). In the presence of the competitive primer analogues, thirteen out of twenty samples (65%) generated specific target product. This is approximately the result that should be predicted by Poisson distribution if the cell

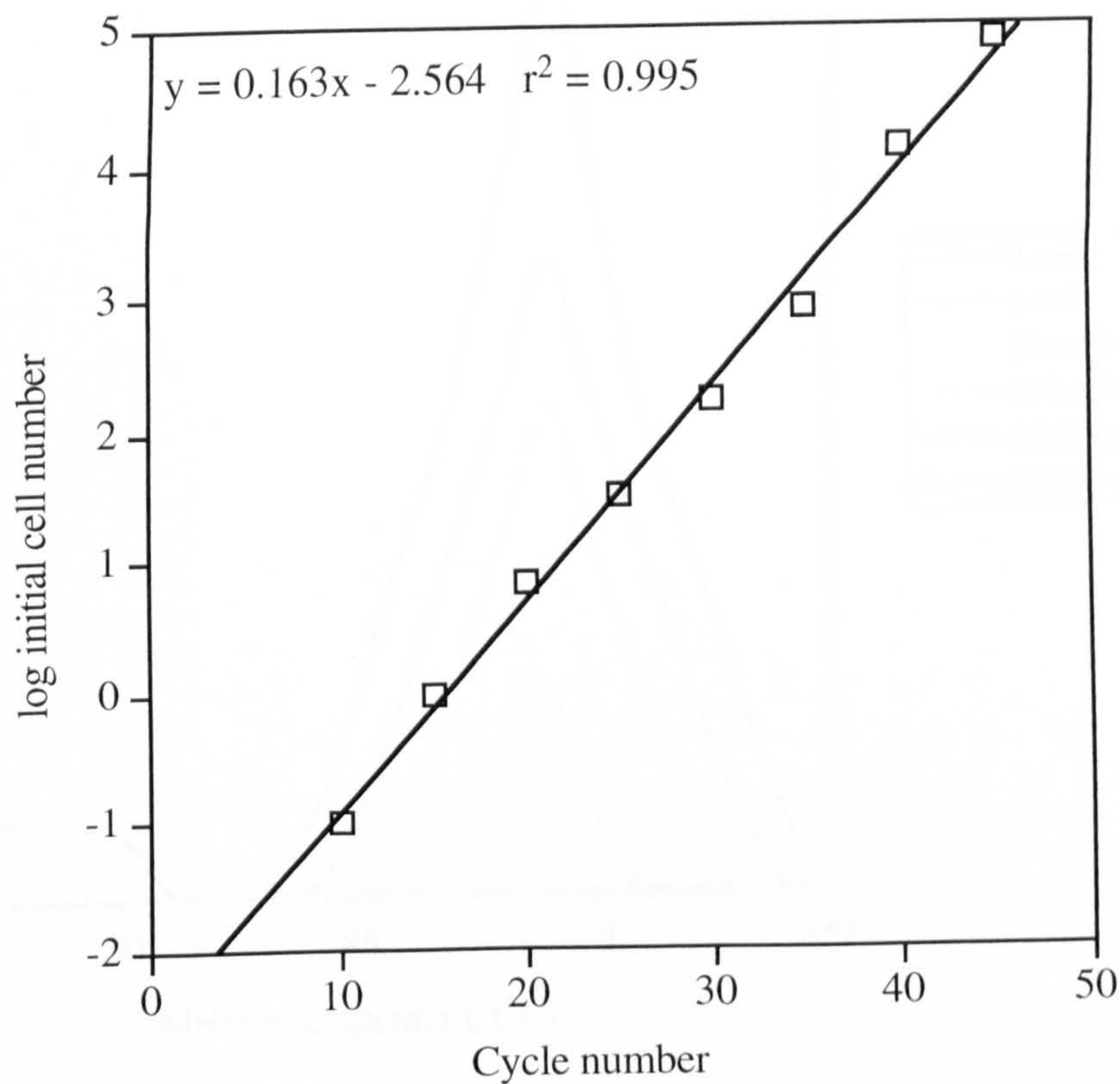


Figure 7.1 Standard graph for the quantification of *Legionella pneumophila*. Samples were amplified for 45 cycles using the standard methodology described in Chapter 2.16.4.

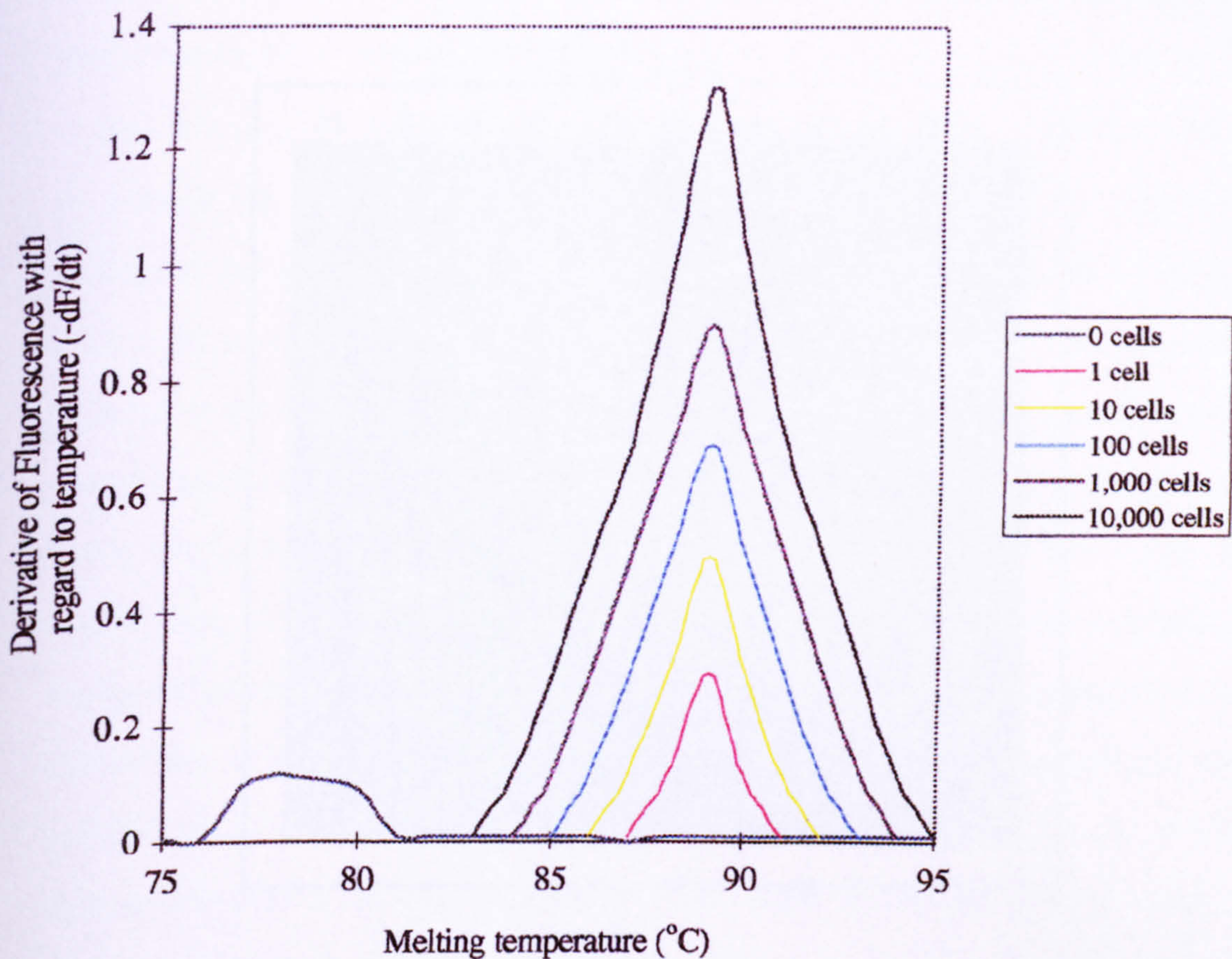


Figure 7.2 Melting curve analysis of products generated from the amplification of known cells of *Legionella pneumophila* in the presence of competitive primer analogues. Analysis of the melting peaks confirmed the purity of the products generated. Melting curves were obtained after 30 seconds of annealing at 74°C, followed by a 0.1°C s⁻¹ linear temperature transition to 94°C.

contained 1 cell of the target (Figure 7.3). In some instances the signals generated were very weak, consequently radioactively labelled probes were used to confirm the number of positive results obtained in some experiments (Section 2.10). This analysis was conducted on twenty five separate occasions to confirm the validity of the results.

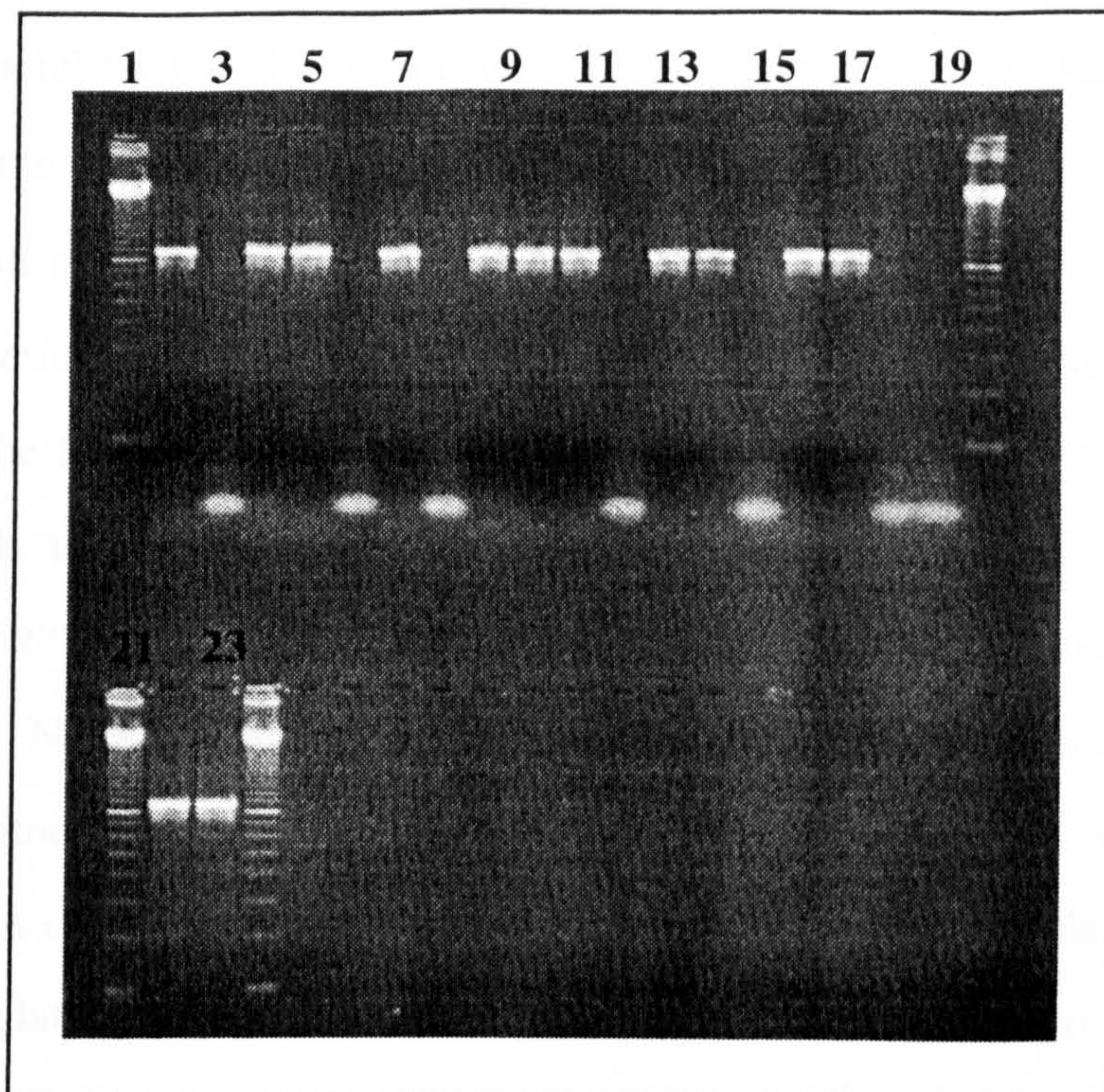


Figure 7.3 Agarose gel analysis of PCR products generated from reactions containing 1 cell of *Legionella pneumophila* in the presence of competitive primer analogues, using the LightCycler™. Lanes 1, 20, 21 and 24, 100 bp ladder; lanes 2-18 and 22-23, replicate reactions containing 1 cell of *Legionella pneumophila*.

7.3 Discussion

The use of 5'-degenerate competitive primer analogues (3'-end labelled with DIG) could theoretically anneal to the binding sites of the functional primers thus reducing the amount of specific product generated. However, with all degenerate primers used in this study, the sequence degeneracy was concentrated at the 5'-end of the primer, because the 3'-end is crucial for the ability to prime and hence compete with the functional primer. As a result, the proper design of these 5'-degenerate, 3'-end labelled competitive primer analogues did not effect amplification efficiency (Table 7.1) thus enabling quantitative PCR to be performed. The use of 3'-DIG-end labelled primer analogues as competitive primers of the functional primers, Leg 1 and Leg 2, blocked the amplification of non-specific products allowing the absolute quantification of 1 cell of *Legionella pneumophila* using the LightCycler™ (this was confirmed by analysing samples using Poisson distribution). Non-specific amplification of this kind occurs due to the mispriming of the functional primer in the presence of very low numbers of target. This takes place because differences in duplex melting temperature between specific template:primer and non-specific template: primer complexes may be as little as 0.5°C (Guo *et al.*, 1997). The competitive primer analogues appear to anneal better to the less-specific template sites than the functional primers. These analogues can not be amplified by *Taq* DNA polymerase thus they effectively block the less-specific sites from the functional primers. Thus, non-specific amplification was reduced without affecting the amplification of the specific product.

CHAPTER 8

GENERAL DISCUSSION

8. GENERAL DISCUSSION

8.1 A review of the Aims of the Study and Conclusions

8.1.1 Factors affecting the amplification efficiency of mimics in competitive PCR

The overall aim of this study was to determine the critical factors affecting the quantification of DNA using PCR and to use the findings to develop an assay for the absolute quantification of DNA in selected model systems. Consequently, work was undertaken to improve the accuracy of quantification using a competitive PCR system by investigating critical parameters affecting amplification efficiency. The results obtained showed that localised sequence domains possessing high T_m s could, under certain circumstances, act as permanent termination sites preventing the amplification of a mimic in competitive PCR. As far as we are aware this is the first time that this phenomenon has been reported. This is of great importance as any perturbation in amplification efficiency resulting from these termination sites could have a profound effect on the relative amplification efficiency of a target and mimic (discussed in Section 3.3). In PCR amplification systems, the potential for variation in amplification efficiency is shown to be large under certain circumstances (McCulloch *et al.*, 1995). The use of *Taq* DNA polymerase, in particular, exacerbated the problem since pausing appears to be followed by a template-independent polymerase activity resulting in the addition of a deoxyadenosine base (Clark, 1988; Clark *et al.*, 1994; Hu, 1993). The resultant mismatched 3'-bases were not extendible by the polymerase since the enzyme has no 3'-5' exonuclease activity. In such circumstances, pause sites may become termination sites with the partial extension products being removed from the amplification process. This leads to a reduction in amplification efficiency which, in a competitive PCR system, would result in a marked difference in yield resulting from relatively small differences

in amplification efficiencies. It has been shown in this work that when designing a competitive PCR, maintaining the minimal feasible difference in size and GC content between the target and mimic may not be sufficient to ensure comparable amplification efficiencies. Equally, careful design of a mimic to avoid pause sites may not be beneficial unless the target can also be assured to lack similar pause sites. The use of betaine should prove to be of great value in competitive systems (where quantification requires equal amplification efficiencies) since it exerts an isostabilising effect on the differential thermal stability of A:T and G:C base pairs eliminating the effect of the high T_m domains.

Other systems which may benefit include those where balanced product yields are required such as multiplex PCR or where high amplification efficiencies are required, such as in the amplification of long DNA templates, where the probability of incorporating a mismatched base is greater than in a PCR using much shorter templates (Barnes, 1994). The use of betaine and/or proof reading enzymes may prove to have general benefits for a range of PCR applications and should perhaps be considered by laboratories seeking to ensure the highest levels of quality and confidence are achieved in the results obtained.

8.1.2 The use of CE and IP-RP-HPLC to determine the suitability and accuracy of competitive PCR for quantification

This study was undertaken to determine the suitability and level of accuracy that could be achieved using the competitive PCR system developed previously by employing either IP-RP-HPLC or CE to quantify target by measuring the point at which an initial amount of mimic added to a PCR generates equal molar amounts of both target and mimic products (the equivalence point) (discussed in Section 4.3).

Accurate quantification using PCR is essential if the full potential of this technique is to be achieved. The amplification-based nature of PCR means that any errors introduced

into the system also become amplified, potentially leading to high levels of variation and inaccuracy. The ability to quantify DNA such as PCR products with high levels of precision and accuracy, as demonstrated here by CE and IP-RP-HPLC, should yield valuable information on the critical points for experimental accuracy. Whilst it is not envisaged that CE or IP-RP-HPLC could replace agarose gel electrophoresis in all instances, they may have a valuable role to play as high metrology standards by which other routes of quantification could be compared in order to determine their fitness for purpose. An advantage that emerged from the application of these two systems to competitive PCR was the ability to monitor heteroduplex formation using a target and mimic with very similar sequences. This investigation demonstrated the ease with which heteroduplexes could form between a target and a mimic. However, both CE and IP-RP-HPLC analysis could potentially be used to correct for any errors generated by heteroduplex formation (Boer and Ramamoorthy, 1997).

In conclusion, this work showed that these two techniques could provide valuable information in the identification and elimination of sources of error and lead to improvements in speed, accuracy and precision, as well as ease of quantification by PCR.

8.1.3 On-line fluorescence monitoring of amplification for the quantification of DNA

Attempts to quantify DNA using mimics of similar and very different sizes, by CE and IP-RP-HPLC, revealed concerns associated with each of these particular approaches (discussed in Chapter 4). Consequently, another approach was adopted for the quantification of target DNA.

The on-line fluorescence monitoring of PCR proved to be a powerful tool for the quantification of bacteria such as *Legionella pneumophila*. This technique was highly reproducible and could be reliably used to detect ten cells. The addition of *AmpliTaq Gold*[™] DNA polymerase to reactions (Section 5.2.3) and the selective measurement of

specific product increased the sensitivity of the assay by an order of 1 magnitude, potentially allowing the detection of a single cell per reaction (Section 5.2.5). The acquisition of melting curve peaks during continuous fluorescence monitoring also allowed the differentiation and quantification of competitive PCR products (Section 5.2.1).

Advances in kinetic PCR, using technologies such as the Lightcycler™, have allowed breakthroughs in the quantification of PCR products. However, the development of similar technologies such as the ABI Prism® 7700 Sequence Detection Systems have allowed specific target to be detected in the presence of non-specific amplification products. Under certain circumstances, the detection of a single molecule has been shown to be possible (Lockey *et al.*, 1998). Unfortunately, the cost of this system is very high, limiting their uptake in routine research and diagnostic laboratories. In contrast, analysis of amplification products using the Lightcycler™ was very rapid and the technology itself considerably cheaper than the other more costly alternative available currently.

The results obtained in Chapter 5 showed that the formation of non-specific products by mispriming were a major problem for the absolute quantification of DNA using the Lightcycler™. In order to develop a method capable of reducing mispriming to improve assay sensitivity, a model system was developed to investigate methods to prevent mispriming and the subsequent generation of non-specific products. The 3'-end labelling of degraded calf thymus DNA and microwaved beef DNA with DIG prevented short DNA fragments from mispriming (and consequently extending) allowing the amplification of a 600 bp target (Section 6.2). These results demonstrated that this strategy could be used successfully to prevent mispriming.

This approach, adapted to produce 5'-degenerate, 3'-DIG-terminated competitive primer analogues (discussed in Chapter 7), coupled with the use of the Lightcycler™, allowed

the detection and absolute quantification of a single cell of *Legionella pneumophila* thus providing a very rapid and sensitive assay for the detection and quantification of microbial pathogens.

The examination of water samples for the presence and number of micro-organisms is basic to environmental microbiology. However, none of the methods in common use permits the determination of exact numbers and types of micro-organisms in an environmental sample. Although some methods of analysis are better than others, every method has certain inherent limitations associated with its use.

The methods described here are suitable for the specific detection and quantification of waterborne bacterial pathogens such as *Legionella pneumophila*. These techniques are generic, and providing sequence information is available for the design of specific primers, the techniques could be adapted to detect and quantify any bacteria. In this instance, the direct amplification of *Legionella pneumophila* from samples was possible immediately after cell lysis due to the lack of inhibitory factors present in tap water. However, if inhibitory factors are present in a sample (as would be expected from sewage or other sources of contaminated surface waters), then extensive purification of DNA following cell lysis may be necessary for successful PCR.

This type of assay would be an effective method of monitoring water supplies contaminated after treatment, and could also be used to assess the effectiveness of water treatment processes by quantifying enteric pathogens such as *E. coli*. Investing in the development of such new screening methods that are more rapid and informative will facilitate the introduction of comprehensive water screening programs. Furthermore, when an outbreak of disease occurs the microbial pathogen responsible and the route by which it was transmitted could be detected and quantified quickly in order to contain the outbreak and treat patients.

In certain other circumstances there are situations when knowledge on the number of cells is also essential. For example, information on the survival of *Mycobacterium tuberculosis* following antibiotic treatment is needed to assess the progress of this debilitating disease. Culture techniques take at least 10 days for growth to become apparent, often 6 to 8 weeks. Therefore, it is possible that the methods described could provide information on the cell numbers so that an early indication of the success of antibiotic treatment can be established.

The advantage of real-time acquisition of data over end-point detection techniques is obvious. Fewer processing steps reduce the opportunity for error. Moreover, the time and effort required by these manipulations are substantially reduced. The quantitative ability of this detection method comes from the ability to monitor the accumulation of amplification products using an intercalating dye. By itself this approach does not control for variables present in the PCR reaction, however it does logically lead toward a technique that could possibly represent the best way to quantify DNA at the moment.

Combined, the use of continuous fluorescence monitoring coupled with the use of competitive PCR mimics could consolidate the best of both methodologies whilst reducing the disadvantages of the individual approaches (Gibson *et al.*, 1996). The inclusion of DNA mimics in reactions monitored continuously by real-time technologies such as the Lightcycler™ serves to control for the inherent variability of PCR. Since recent advances allow the use of multiple fluorogenic probes in a single reaction (Williams *et al.*, 1998), the differentiation of mimic and target by DNA melting curves could detect both a target and a DNA mimic simultaneously.

Although a great variety of competitive PCR protocols have been developed for the precise quantification of a target they all involve mixing varying amounts of mimic, usually differing in size from the target product, with a constant amount of sample to be determined (discussed in Section 1.3.2). This requires multiple PCR amplifications in

order to obtain an accurate determination of the target concentration in a regression plot. The amount of labour required to perform this type of analysis could hamper the large-scale routine quantification of samples. The use of multiple competitive mimics in a single tube, coupled with technologies such as the Lightcycler™, could provide an even more rapid and accurate method to address the problems associated with this form of analysis. A number of researchers have already used this approach successfully for the quantification of HIV-1 (Zimmermann *et al.*, 1996a and 1996b).

More recently, advances in the miniaturisation of fluidic and sensing components is making possible fully integrated, miniaturised diagnostic systems and it is in this area that the next generation of systems for quantifying DNA will emerge in the form of "DNA chips" (Ibrahim *et al.*, 1998; Northrup *et al.*, 1998; Petersen *et al.*, 1998; Waters *et al.*, 1998). However, the use of PCR is still fundamental to these systems for the amplification of target DNA. The conclusions presented in this work concerning PCR for the quantification of DNA still apply to this type of miniaturised system. For instance, the need to design mimics carefully to avoid pause sites.

Miniaturisation offers the promise of rapid, on-site quantification (Belgrader *et al.*, 1998). Real-time diagnosis of diseases such as chlamydia has tremendous value. Obtaining a test result while the patient is still in the office allows for immediate treatment or counselling decisions. Furthermore, testing candidate blood donors for viral pathogens before they give blood would save the blood product industry time and money associated with pooling and processing blood that is later found to be contaminated. Donor testing could also improve the safety of the blood supply for transfusion medicine, especially during times of serious donor blood shortages when rapid donor campaigns are under way.

Incidences involving significant morbidity and mortality due to the contamination of water supplies or foodstuffs by bacterial pathogens (such as *E. coli* 0157) point out the

need for fast, easy, on-site testing. With a portable system, it would be possible to analyse samples for the presence of microbial pathogens, thus determining the level of contamination and preventing their introduction into water or food processing plants. This would also offer the ability to immediately identify and trace sources of infection or pollution.

Therefore, it is within the area of small, portable, self diagnostic systems that quantifying DNA will have the greatest future impact (Belgrader *et al.*, 1998; Belgrader *et al.*, 1999). Individuals will not only be able to detect microbial pathogens (e.g. *Yersinia pestis*) using these systems but also determine the level of infection. The data could then be sent to a doctor allowing remote diagnosis of disease. This would be of great benefit in remote areas where medical facilities are severely limited or non-existent.

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APPENDIX I

Appendix I

Composition and use of microbiological media

Media	Composition (per litre)	Use
2 x LB media	6 g select tryptone 140, 5 g select yeast extract and 10 g NaCl was made up to 1 L with ddH ₂ O. Before use, antibiotic (if required) was added.	The propagation of all bacterial strains.
SOC	4 g select peptone 140, 1 g select yeast extract, 0.1 g NaCl and 250 mM KCl, pH 7.5 (2 ml) was made up to 200 ml with ddH ₂ O. After autoclaving, 1 M filter-sterilised glucose (4 ml) was added when the broth had cooled to 50°C. Prior to use, 2 M MgCl ₂ (1 ml) was added.	The propagation of all <i>E. coli</i> cells after transformation.

APPENDIX II

Appendix II

Antibiotics used for strain-specific selection.

Antibiotic	Action	Preparation	Application
Ampicillin	Inhibits the final stages of peptidoglycan synthesis, resulting in a weakening of the bacterial cell wall.	Ampicillin (sodium salt) was dissolved in ddH ₂ O to a final concentration of 50 mg/ml, and sterilised by filtration through a 0.22 µm acrodisc (Gelman Sciences). Stock solutions were stored at -20°C.	0.1 mg/ml in 2 LB broth/agar for the selection of <i>E. coli</i> .

APPENDIX III

Appendix III

Preparation of buffers and solutions

Buffer/Solution	Method of Preparation
10 x Agarose gel loading dye	2 ml Glycerol (40% v/v), 0.5 ml (5%) Bromophenol Blue (0.5%), 0.5 ml (500 mM) EDTA (50 mM) plus 0.5 g SDS [10% w/v] was adjusted to 5 ml with ddH ₂ O.
Agarose gels	<p>1%: 1 g agarose was melted per 100 ml 1 x TBE. The molten gel was cooled to 50°C prior to the addition of EtBr (0.5 µg/ml final concentration). The molten gel was poured into a gel casting tray and allowed to set for at least 30 minutes. before use.</p> <p>1.75%: as above, except 1.75 g was melted per 100 ml 1 x TBE.</p> <p>2%: as above, except 2 g was melted per 100 ml 1 x TBE.</p>
10% ammonium persulphate (APS)	0.1 g APS was dissolved in 1 ml ddH ₂ O. 10% APS was prepared freshly each time.
50 mM CaCl ₂	2.7 g CaCl ₂ .6H ₂ O was dissolved in 200 ml ddH ₂ O. The solution was sterilised by autoclaving.
Chloroform-IAA (24:1)	40 ml IAA was added to 960 ml chloroform. Chloroform-IAA was stored at RT protected from light.
50 x Denhardts solution	2 g Ficoll [1% V/V], 2 g PVP [1% W/V] and 2 g BSA [1% W/V] were dissolved in 200 ml ddH ₂ O, filter sterilised and stored at -20°C.
Denaturing solution	200 g NaOH (500 mM) plus 584 g NaCl (10 M) was dissolved in 10 L ddH ₂ O.
500 mM DTT	0.77 g DTT was dissolved in 10 ml ddH ₂ O and stored at -20°C.
0.5 M EDTA (pH 8.0)	186.1 g EDTA was dissolved in 800 ml ddH ₂ O and the pH adjusted to 8.0 with 10 M NaOH. The final volume was adjusted to 1 L with ddH ₂ O and sterilised by autoclaving.
EtBr (5 µg/ml)	0.1 g EtBr was dissolved in 10 ml ddH ₂ O. EtBr solutions were stored at RT protected from light, and used at a working concentration of 0.5 µg/ml in agarose gels and/or 1 x TBE buffer.
Filter washing solution	Diluted from 20 x SSC and 10% SDS stock solutions, as follows; 0.20 x SSC/0.1% SDS. The filter washing solution was stored at room temperature.

Herring sperm DNA	Herring sperm DNA (Sigma); 100 mg were dissolved in 10 ml sterile water with shaking overnight. This was warmed for 1 hour at 37°C and then cooled. The DNA was sonicated for 3 x 2 minute bursts with 1 minute intervals, then transferred to a stoppered glass tube and boiled for 10 minutes, before being cooled on ice and distributed in 250 µl aliquots. Stored at -20°C.
10 x High stringency wash buffer	8.77 g NaCl, 4.41 g Na Citrate (0.2 x SSC) plus 10 g SDS (1% w/v) was adjusted to pH 7.75 with HCl and to a final volume of 1 L with ddH ₂ O.
Hybridisation Buffer	5 ml Formamide (20%w/v), 6.25 ml 20 x SSC (5 x SSC), 0.25 ml 50 x Denhardts (1 x), 1 ml 500 mM NaPO ₄ (20 mM) plus 0.25 ml 10 mg/ml Boiled Herring Sperm DNA (0.1 mg/ml) were made upto a final volume of 20 ml with ddH ₂ O.
IPTG (0.2 µg/ml)	2 g IPTG was dissolved in 10 ml ddH ₂ O and sterilised by filtration through 0.2 µm acrodiscs (Gelman Sciences). Stock IPTG was stored in 1 mls aliquots at -20°C.
10 x Kinase buffer	500 µl 1 M Tris-HCl pH 8.0 (500mM), 60 µl 1 M MgCl ₂ (60mM), 50 µl 100 mM DTT (5mM), 125 µl 2M KCl (250mM) plus 20 µl 50 mg/ml BSA (1mg/ml) were adjusted to a final volume with 245 µl ddH ₂ O and filter sterilised.
10 x Ligation buffer	1320 µl 1 M Tris-HCl pH 7.4 (660 mM), 30 µl 500 mM MgCl ₂ (66 mM) plus 400 µl 500 mM DTT (100 mM) were made up to a final volume with 253 µl ddH ₂ O, filter sterilised, and stored at -20°C.
Molecular weight markers	100 µl of 1 Kb DNA molecular weight marker (or 100 bp molecular weight marker) was added to 800 µl 1 x TBE and 100 µl 10 x Agarose gel loading dye. Stock molecular weight markers were stored at -20°C and 5 µl routinely loaded per well.
2.5 M NaOAc (pH 5.2)	81.62 g NaOAc was dissolved in 150 ml ddH ₂ O and the pH adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 200 ml with ddH ₂ O, and sterilised by autoclaving.
10 M NaOH	400 g NaOH dissolved in 1 L ddH ₂ O and filter sterilised.
500 mM NaH ₂ PO ₄	60 g NaH ₂ PO ₄ was dissolved in 1 L ddH ₂ O and filter sterilised.
Neutralisation solution	605 g Tris pH 7.4 (500 mM), 876 g NaCl (1.5 M), 400 ml Conc. Hcl were adjusted to a finsal volume of 10 L with ddH ₂ O.
10 x PCR buffer	25 ml 1 M KCl (50 mM), 0.37 g Tris base, 0.307 g Tris HCl (10 mM Tris pH 8.3), 0.05 g Gelatin (0.01%) were adjusted to a final volume of 50ml with ddH ₂ O and filter sterilised.
Phenol-chloroform (1:1)	Equal volumes of TE-equilibrated phenol and chloroform were added. Phenol-chloroform was stored protected from light at 4°C

Appendices

10% SDS	100 g SDS was dissolved in 800 ml ddH ₂ O and the final volume adjusted to 1 L with ddH ₂ O.
20 x Standard saline citrate (20 x SSC)	175.3 g NaCl and 88.2 g Na citrate were dissolved in 800 ml ddH ₂ O. The final volume was adjusted to 1 L with ddH ₂ O and the solution sterilised by autoclaving.
10 x Tris-borate EDTA buffer (TBE);	216 g Tri (0.045 M), 110 g Boric Acid plus 20 ml 0.5 M EDTA (pH 8.0 1mM) wer made up tp a final volume of 10 L with ddH ₂ O.
T ₁₀ E ₁	10 ml 1 M Tris (pH 7.5) plus 2 ml 500 mM EDTA (pH 8.0) were added to 1 L ddH ₂ O and filter sterilised.
TE equilibrated phenol	1 kg phenol detached crystals was dissolved in 1 L of 1 M Tris-HCl, 1 mM EDTA, pH 8.0 and stirred overnight at 4°C. The top layer was removed by aspiration and 1 L of 0.5 M Tris-HCl, 1 mM EDTA, pH 8.0 added. 0.1% (w/v) 8-Hydroxyquinoline was added to help prevent oxidation of phenol which occurs at RT or on exposure to light. TE-equilibrated phenol was stored at 4°C.
TE pH 8.0	10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0).
10 x TBE	108 g Tris base, 55 g boric acid and 40 ml 0.5 M EDTA (pH 8.0) was dissolved in 800 ml ddH ₂ O. The final volume was adjusted to 1 L with ddH ₂ O. TBE was used at a working strength of 1 x (i.e., a 1:10 dilution of the concentrated stock) for polyacrylamide gel electrophoresis.
1 M Tris-HCl (pH 8.0)	121.1 g Tris base was dissolved in 800 ml ddH ₂ O, and the pH adjusted to 8.0 with HCl. the final volume was adjusted to 1 L with ddH ₂ O and the solution sterilised by autoclaving.
X-gal (20 mg/ml)	20 mg X-gal was dissolved in 1 ml dimethylformamide. Stock X-gal was stored at -20°C protected from light.

APPENDIX IV

Appendix IV

Source and catalogue numbers of general chemicals

Chemical	Source	Catalogue No.
1 Kb molecular weight marker	Gibco BRL	15615-024
100 bp molecular weight marker	Pharmacia Biotech	27-4001-01
Acetic Acid (Glacial)	BDH AnalaR	Cat. No.10001
Adenosine Triphosphate Tetra(triethylammonium) salt [α - 32 P](555 kbq,111 Tbq/mmol)	Amersham	Cat. No. PB10168
Agarose	Gibco BRL	Cat. No. 540- 5510UB
Albumin (Bovine)	Sigma	A7030
Ammonia (NH ₃)	BDH AnalaR	Cat. No. 10012 67
Ampicillin sodium salt	Sigma	A-0166
Bacto-Agar	Difco	Cat. No. 0140- 01-0
Bacto-Tryptone	Difco	Cat. No. 0124- 01
Bacto yeast extract	Difco	Cat.No. 0127- 01
β -Fluor	National Diagnostics	Cat. No. LS- 151
β -Mercaptoethanol	Sigma	M3148
Bromophenol Blue	Sigma	B5525
Calcium Chloride (CaCl ₂);	Fisons	C/1360/53
Chloroform	Rathburn Chemicals Ltd.	RH1049
Citric Acid (Trisodium dihydrate)	Sigma	C8532
Deoxyadenosine Triphosphate Tetra(triethylammonium)salt [α - 32 P] (555 kbq,111 Tbq/mmol)	NEN Dupont	Cat. No. 33002X
Deoxycytidine Triphosphate (dCTP)	Boehringer Mannheim	Cat. No. 1051- 458

Deoxygaunosine Triphosphate (dGTP);	Boehringer Mannheim	Cat. No. 1051- 466
Deoxythymidine Triphosphate (dTTP)	Boehringer Mannheim	Cat. No. 1051- 482
Dimethyl Sulphoxide (DMSO)	BDH AnalaR	Cat. No. 10323
Dithiothreitol (DTT)	Sigma	D0632
EDTA (ethylenediaminetetraaceticacid)	Sigma	EDS
Ethidium Bromide (EtBr)	Sigma	E-2515
Ethanol (EtOH)	Fisher	E/0650DF/17
Formamide	BDH AnalaR	Cat. No. 10326-4R
Film (20x40cm)	Fuji	Cat. No. 036250
Glycerol	Sigma	G-7893
Herring sperm DNA; Sigma, D3159	Sigma	D3159
Hydrochloric acid (HCl) sp.gr.1.18	BDH	101256J
IAA (isoamyl alcohol)	Sigma	I-9392
IPTG (isopropyl-β-D-thiogalactopyranoside)	Sigma	I-5502
Isopropanol (propan-2-ol)	Sigma	I405-7
KCl (Potassium Chloride)	Fisons	P/4280/53
KH ₂ PO ₄ (Potassium Dihydrogen Phosphate)	Sigma	P5504
KOAc (Potassium Acetate)	Sigma	P-3542
Kodak x-ray developer (LX24)	Kodak	Cat. No. 328091C
Kodak Unifex	Kodak	Cat. No. 328278
MgCl ₂ (Magnesium Chloride)	Sigma	M-2393
MgSO ₄ (Magnesium Sulphate)	Sigma	M-2643
Molecular Weight Markers (λ DNA/HindIII)	Gibco BRL	Cat. No. 520- 561 2SA
Phenol detached crystals	Fisher	P/2360/60
Select agar	Gibco BRL	30391-015
Select yeast extract	Gibco BRL	30393-029
Sodium Acetate (Anhydrous)	Sigma	S-8750
Sodium Azide	Sigma	S-2002

Sodium Carbonate (Anhydrous)	BDH AnalaR	Cat. No. 10240
Sodium Chloride (NaCl)	Sigma	T525
triSodium Citrate	BDH AnalaR	Cat.No. 10242-5M
diSodium diHydrogen Orthophosphate (Na ₂ H ₂ PO ₄)	BDH Aristar	Cat. No. 45232 1C
SDS (sodium dodecyl sulphate)	Sigma	L-5750
Sodium Hydroxide pellets (NaOH)	Sigma	S-5881
Sodium Perchlorate (Anhydrous)	Sigma	S1513
Sodium Phosphate (NaPO ₄)	Sigma	S8282
Spermidine	Sigma	S2626
Sulphuric Acid (H ₂ SO ₄);	BDH Aristar	Cat. No. 45006
Tris base [Tris(hydroxymethyl)aminomethane]	Sigma	T-1503
Tris-HCl [Tris(hydroxymethyl)aminomethane hydrochloride]	Sigma	T-6666
Trichloroacetic Acid (TCA)	BDH	Cat. No. 10286
X-gal (5-bromo-4-chloro-3-indolyl β-D- galactopyranoside)	Sigma	B-9146

Supplier

Sigma Immunochemicals, Poole, Dorset, UK.

Pharmacia Biotech, St. Albans, Herts, UK.

Gibco BRL Life Technologies Ltd., Paisley, Scotland, UK.

Rathburn Chemicals Ltd., Walkerburn, Scotland. UK.

Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK.

Perkin Elmer Applied Biosystems Intl., Cheshire, UK.

BDH Merck Ltd., Poole, Dorset, UK.

Fisher Scientific U. K., Loughborough, Leics., UK.

Difco Laboratories Ltd., West Molesey, Surrey, UK.